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# QUALITY CONTROL OF PROTEIN AND PEPTIDE DRUGS: monoclonal antibodies and some biological response modifiers derived by recombinant DNA technology

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## ABSTRACT

Monoclonal antibodies have found a wide and varied use in many areas of biomedical research, such as diagnosis, tumour imaging and immunopurification, and some of these protein products are already in use or will soon be used as therapeutic agents. Products derived by recombinant DNA technology, for instance biological response modifiers, offer prospects to develop new medicines. As for all other drugs, the quality of biotechnological products, intended for use in man or animal, should be established. Because of the complex nature of these proteins, when compared with a conventionally produced chemical product, and the production process, which may introduce several contaminants which are hard to detect, in-process control is a prerequisite. In order to understand the in-process controls, the theory of monoclonal antibodies as well as the theory of recombinant DNA technology is reviewed. Subsequently, the quality control and the validation of these

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tests are discussed. The main risk factors, being the contamination with DNA and viruses, and the purity and identity of the final product are underlined.
As supplement, the main biological and biochemical characteristics of the interleukins, tumour necrosis factor, interferons, and colony stimulating factors are reviewed.



## 1 INTRODUCTION

Biotechnological products may be roughly classified in three categories:

1. those obtained by the fusion of homologous or heterologous cells, hybridomas, from which monoclonal antibodies are derived,
2. those obtained by recombinant DNA technology, and
3. those derived from transformed continuous cell lines.

Monoclonal antibodies have found a wide and varied use in many areas of biomedical research such as diagnosis, tumour imaging and immunopurification. They are also used in cancer therapy. Products obtained by recombinant DNA technology or derived from transformed continuous cell lines, such as interferons and interleukins, are already in use or will soon be used as therapeutic agents. As for all other drugs, the quality of biotechnological products intended for use in vivo must be established. Normally, the final product is a complex protein, eventually glycosilated or linked with disulphide bonds, and is not easy to analyse when compared to a conventionally produced chemical product. Moreover, the production process may introduce contaminants, which are hard to detect. This indicates the importance of in-process control as well as methods for the validation of these tests and of purification methods. Such in-proces tests, which are an integral part of the control of the final product, are essential. In order to understand the in-process controls, the theory of monoclonal antibodies is reviewed in chapter 2. The quality control of monoclonal antibodies and the validation of the tests used are outlined in chapter 3. In addition, the production of medicinal products derived by recombinant DNA technology and its quality control will be discussed in chapter 4. As an appendix, the main characteristics of some biological response modifiers, which are derived by recombinant DNA technology, are summarized in chapter 5. A general discussion is given in the last chapter.

## 2 MONOCLONAL ANTIBODIES

### 2.1 ANTIBODY STRUCTURE AND FUNCTION

The immunoglobulins (Ig), or antibodies, are a group of glycoproteins present in the serum and tissue fluids of all mammals. Their production is induced when the host lymphoid system comes into contact with foreign molecules (antigens) and they bind specifically to the antigen which induced their formation. They are, therefore, an element of the adaptive immune system. To start the immune response, an antigen is phagocytized by a macrophage, and the macrophage places parts of the antigen on its surface membrane. A T-lymphocyte then recognizes the antigen and the macrophage. In turn, it stimulates B-lymphocytes, via soluble mediators, the so-called lymphokines, to produce antibodies to the antigens. Each B-lymphocyte contains many copies of the antibody molecule on its surface. When an antigen binds to a surface antibody the B-lymphocyte begins to divide rapidly, producing millions of copies of itself. These mature B-lymphocytes secrete large quantities of the single specific antibody.

In general, antibodies are symmetrical molecules made up of two identical glycosylated heavy chains of a relative molecular weight (Mw) of 50 kiloDalton (kD) to 75 kD, and two identical non-glycosylated light chains of Mw about 25 kD. The heavy chains are joined by disulfide bonds to each other, and each light chain is joined by a disulfide bond to one heavy chain.

Five distinct classes of immunoglobulin molecules are recognized in most higher mammals, namely IgG, IgA, IgM, IgD and IgE. These differ from each other in size, charge, amino acid composition and carbohydrate content. In addition to the differences between classes the immunoglobulins belonging to each class are also very heterogenous.

The class of an immunoglobulin molecule is determined by its heavy chains. Thus IgG, IgA, IgM, IgD and IgE posses gamma, alpha, mu, delta and eta heavy chains respectively. The light chain kappa or labda is characteristic of this whole group of proteins.

IgG is the major immunoglobulin in normal human serum accounting for 70-75% of the total immunoglobulin pool. IgG is a monomeric protein, with a Mw of 160 kD and can be divided in four subclasses IgG1 - IgG4, with heavy chains called gamma 1-4. Its carbohydrate content is 2-3%.

IgA represents 15-20% of the human serum immunoglobulin pool. Human IgA exists for 80% as the basic four chain monomer but in most mammals the IgA in serum is mainly polymeric. There are two subclasses, IgA1 and IgA2, with heavy chain alpha 1 and 2 respectively. The carbohydrate content is about 7-11%, the molecular weight is 170-500 kD.

IgM accounts for about 10% of the immunoglobulin pool. The molecule has a pentameric structure in which individual heavy chains (mu-type) have a molecular weight of approximately 65 kD. The carbohydrate content is about 9-12%.

IgD accounts for less than 1% of the total plasma immunoglobulin, but is known to be present in large quantities on the membrane of many circulating B-lymphocytes. Its molecular weight is about 70 kD, its carbohydrate content 12-15%.

IgE is found on the surface membrane of basophils and mast-cells in all individuals and as a trace in serum. Its molecular weight is 80 kD and its carbohydrate content is 12% [1-3].

The secondary and tertiary structure within one class are the same, but the differing primary sequence in the variable and hypervariable regions will alter the different molecules: net charge, net density and hydrophobicity. The nature and quantity of the carbohydrate moiety will also affect these characteristics [4].

## 2.2 DEVELOPMENT OF MONOCLONAL ANTIBODIES

When two cells are brought into close contact and their membranes caused to fuse toghether, the resulting fusion contains both nuclei. A cell with two or more dissimilar nuclei is called a heterokaryon and in due course the nuclei can fuse together producing a single nucleus with genetic information from both of the orginating cells. This fusion is called a hybrid. In 1975 Kohler and Milstein [5] fused antibody-producing mouse spleen cells with mouse myeloma cells. The hybrids they obtained secreted antibodies of the specificity dictated by the parent spleen cell, but in quantity characteristic of a myeloma [6]. Monoclonal antibodies are produced by a clonal population of lymphocytes. Most are of the IgG-type, though IgM and IgA have also been produced for commercial purposes [1,4].

### 2.2.1 Production

The procedure for producing monoclonal antibodies (Mab's) begins with the injection of the antigen into an animal. The mouse is usually the animal of choice, because suitable mouse myelomas are widely available, and growth of hybridomas in

mice is usually no problem. All of the currently available mouse myeloma lines, which are suitable for fusion, are of BALB/c origin [3,7]. Other animals used for antibody production are rats, rabbits, goats, sheep and even horses. In traditional antiserum techniques the antigen needs to be highly purified. In the monoclonal antibody approach, purification is not essential, but it is useful [7]. Apart from the in vivo immunization, in vitro immunization procedures are developed, which are performed when antigen is severely limiting or when a fusion with a human myeloma is required [1,7]. Many immunization protocols have been utilized [6].

In 1975, Kohler and Milstein [5] described a general method for the production of homogeneous antibodies of predefined specificity. They fused the hypoxanthine, aminopterin, and thymidine (HAT) sensitive variant of MOPC-21 myeloma cells with spleen cells from mice, immmunized with sheep red cells. The fusion was mediated by Sendai virus, and hybrids were selected by growth in HAT medium. It was known that normal spleen cells could only survive a few days in culture, and myeloma cells appear to be "immortal". The normal purin and pyrimidin synthesis is blocked by aminopterin. By adding hypoxanthine and thymidine, the salvage pathway is starting up by the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT), and thymidine kinase (TK).

Mutant myeloma cells lacking HGPRT and/or TK are produced by using toxic drugs, such as thioguanine. If spleen cells, which possess TK and HGPRT but die in culture, are fused with myeloma cells lacking TK and HGPRT, only the hybrid cells will grow in the HAT-medium [3].

The experiment of Kohler and Milstein resulted in a number of cloned hybrid lines secreting anti-sheep erythrocyte antibodies [5]. Alternative selection systems use mutants lacking either adenine, phosphoribosyl transferase or adenosine kinase, or selective sensitivity to the antibiotic amphotericin B methyl ester or to ouabain [6].

The basic procedure is essentially unchanged, but the use of the Sendai virus has been superseded by polyethylene glycol (PEG), which has a higher fusion frequency and greater reproducibility [8]. Other methods which may be useful for hybridoma production are those in which cells are fused in electric fields [1,3].

The efficiency of myeloma spleen cell can be increased by incorporating insulin into the HAT medium [9]. The maximal effect of insulin ranged from $10^{-1}$ and $10^{-4}$ IU/ml, however, if insulin is added, assurances would be needed that it is removed from the final product [3].

After a week in HAT medium it is safe to assume that all parental myeloma cells are dead, and the growing cells are hybrids. The next step in the production of

monoclonal antibodies is to select the hybridoma, which will be cloned. Initially, it is important to identify antibody secretion so a non antigen-specific assay is used, such as a binding assay based on Staphylococcus aureus protein A which is selective for some classes of Ig-antibodies. Then an assay selective for an particular antigen specificity is used. The most commonly employed screening assays are solid-phase radioimmunoassay, enzyme linked immunosorbent assays, immunofluorescence screening and cytotoxicity assay [1,3,7].

The next step in the production process is cloning, which ensures that all of the hybridoma cells are actually of one clonal population. Otherwise, the antibodies secreted by these hybridomas may actually be a mixture of antibodies that recognize different receptor sites on the target antigen. Cells may be cloned by growth in soft agar or using the fluorescence-activated cell sorter [10], but up till now the cloning by limit dilution is preferred [1,3,7]: the hybridomas are diluted, and one cell is deposited into each well of a 96-well culture plate. The single cell grows into a colony or clone of identical hybridoma cells. Single-colony wells are rescreened for specific antibody production, and a hybridoma clone is selected [7]. Usually, the medium is fetal calf serum to which 10% dimethylsulfoxide (DMSO) is added [3].

There are two well established technologies for producing monoclonal antibodies. In the first case, the in vivo method, hybridoma cells are injected intraperitoneally in mice where they proliferate and secrete monoclonal antibodies into the ascites fluid, which is then collected. The other method is an in vitro method: the hybridoma cells are cultured in nutrient media. Two systems can be discerned, namely the homogeneous suspension culture (airlift reactor, continuous stirred reactor) and the immobilized or entrapped cell culture (hollow fiber perfusion [11], microcapsules [12], agarose beads, ceramic beads [13]; see [14]).

### 2.2.2 Purification

The degree of purity required of a monoclonal antibody product depends upon its intended application. The level of purity of the monoclonal antibody at the production stage depends on the method of production. Typical antibody concentrations in serum or ascites fluid of hybridoma bearing mice range from 2-20 mg/ml, and thus represent a significant fraction of all protein present. In contrast, the antibody levels in culture supernatants of hybridomas are in the order of magnitude 5-50 μg/ml. It is therefore likely that purification of antibodies from serum or ascites will be much easier than from culture supernatant [1].

2.2.2.1 Pre-clearance and initial enrichment

The first step following ascites or tissue culture production of monoclonal antibodies is to separate cells and particulates from the fluid. The primary concern here is clarification of the soluble product to increase isolation efficiency. Techniques used to clarify supernatant or ascitic fluid include centrifugation and ultrafiltration. Ultrafiltration can be used for both concentrating and conditioning of protein solutions. If de-salting and buffer exchange are combined with ultrafiltration the method is called diafiltration [4].

2.2.2.2 Precipitation

One of the oldest and most useful methods of purification of immunoglobulins is based on the observation that they are precipitated by lower concentrations of ammonium sulphate than most other serum proteins. Precipitation of immunoglobulins by ammonium sulphate is effective and gentle [1]. Euglobulin precipitation is based on the insolubility of certain proteins in aqueous systems because of their isoelectric condition [16,17,18]. Neoh et al. [19] used polyethylene glycol 6,000 for the purification of mouse monoclonal IgG and IgM from ascitic fluids. Caprylic acid is used to precipitate plasma proteins, monoclonal antibodies are harvested from the supernatant [20].

While it is not possible to purify to homogeneity by either of these methods, it provides a substantial enrichment, and reduces the protein load on subsequent purification steps [1].

To condition the preparation prior to intermediate purification, de-salting and buffer change can be combined with ultrafiltration or gel filtration chromatography can be used [4].

2.2.2.3 Intermediate purification

For the intermediate stage of the process there are three techniques for large scale purification of monoclonal antibodies:

1. adsorption chromatography
2. ion exchange chromatography
3. affinity chromatography [4].

ad 1. Adsorption chromatography

Hydroxyapatite is a crystalline form of calcium phosphate that has been in use since the 1950's for purifying proteins and nucleic acids [21]. Although adsorption

chromatography on inorganic matrices such as hydroxyapatite is still not totally understood, an electrostatic interaction is undoubtedly an important component of the adsorption mechanism [4]. For monoclonal antibodies, it has been suggested that separation is based on variation on the light chain composition [21].

A recent study, comparing anion exchange, hydroxyapatite adsorption chromatography and affinity chromatography (protein A) for purification of three different monoclonal antibodies, found that hydroxyapatite chromatography was the least efficient in terms of yield and purity [18]. Pavlu et al. [23] demonstrated that both ion-exchange (Mono Q HR 515 anion exchange column) and hydrophobic interaction chromatography (TSK gel phenyl 5 PW column) give higher resolution than hydroxyapatite chromatography.

ad 2. Ion exchange chromatography

An ion exchanger consists of an insoluble matrix to which charged groups have been covalently bound. The charged groups are associated with mobile counter ions. These counter ions can be reversible exchanged with other ions of the same charge. In ion exchange chromatography the isoelectric point (pI) and charge density of the monoclonal antibody should dictate the type of ion exchange employed. The affinity of the monoclonal antibody can be controlled by varying conditions such as ionic strenght and pH [4,15].

Ion exchange is a popular method, it is a gentle technique with good yield and a high level of purity is obtained ( in a two-step procedure IgM monoclonal antbodies were purified to >90% purity in 80 minutes , as checked by Sodium Dodecyl Sulfate - PolyAcrylamide Gel Electrophoresis (SDS-PAGE)) [4]. The most commonly used matrix consists of cellulose or agarose attached to which are is attached ionizable diethylaminoethyl (DEAE) groups, being anion exchangers. The antibodies are generally eluted by the addition of competing anions; with increasing concentrations of eluting anion proteins are eluted in order of their isoelectric points [3].

The majority of serum proteins are strongly acidic with isoelectric points below 5.5, while most immunoglobulins are only weakly acidic with isoelectric points above 6. In cation exchange the conditions (pH and ionic strenght) can be manipulated so that all the product and only a few of the contaminants are bound initially, thus eliminating the need for high resolution gradient elution. Cation exchange is, therefore, more suitable than anion exchange when scaling up to gram quantities of product. Yields are more than 80%, and purity is about 50-90% [3].

Mixed mode ion exchange chromatography has been performed on preparative antibody exchanger (ABx) columns. The research done by Ross et al. [24] was focused on monoclonal antibodies, which were known to bind weakly to protein A (murine IgG1 and rat Mab's). The purity of the tested monoclonal antibodies were excellent, especially murine IgG1 (purity >99%).

ad 3. Affinity chromatography

The most principle way to purify monoclonal antibodies is by affinity chromatography. If the antigen against which the antibody is directed is available in a suitable form, it may be coupled to cyanogen-bromide-activated Sepharose beads. Alternatively a specific anti-immunoglobulin antibody can be immobilized. However, there are certain disadvantages associated with these immunoaffinity methods. The binding of antigen to the antibody takes place by the formation of multiple non-covalent bonds between the antigen and amino acids of the binding site. Although the attractive forces (namely, hydrogen bonds, electrostatic interaction, Van der Waals forces, and hydrophobic interactions) involved in these bonds are individually weak compared to covalent bonds, the multiplicity of the bonds leads to a considerable binding energy [2]. Because of these very strong antigen-antibody complex bonds, recovery of the antibody may require rather harsh elution conditions which are likely to destroy the antibody activity and/or disrupt the covalent linking the ligand to the matrix and result in elution of the entire complex. Moreover, for large scale purification, the quantities of antigen or anti-immunoglobulin antibody required for purification are still too large to provide economic purification [1,3,4].

Affinity chromatography using immobilized protein A is a well documented method for research-scale purification of immunoglobulins, especially IgG. Yields are between 50 and 80%, and purity was close to 100% [3,25].

### 2.2.2.4 Final purification

This stage of the process is only undertaken if the demands for product purity are very high, e.g. when the monoclonal antibodies are intended for use in man, and the desired purity has not been achieved with an intermediate purification step. Final purification can also be used to obtain the product in a defined environment. Gelfiltration, sometimes known as size exclusion chromatography, is the technique commonly used for this stage [3]. Gel filtration separates proteins according to their size [26]. A range of gels are available, the main types of gels are granulated dextran (Sephadex), acrylamide (Bio-gel P), agarose (Sepharose, Biogel A) and

various combinations of these. Sephacryl is a mixture of dextran and acrylamide; Ultrogel is a mixture of agarose and acrylamide. The newer columns Superose 6B, Superose 12 and TSK gels are suitable for high performance liquid chromatography [1,27].

Monoclonal antibodies may be purified by other methods including zone electrophoresis, chromatofocussing and isotachophoresis [28]. These techniques are based on the differences in isoelectric points of proteins, but are rarely used for large scale immunoglobulin purification, because these methods are slow and cumbersome, and have a limited capacity. However, particularly zone electrophoresis is capable of excellent separations, and is especially useful for monoclonal IgM and IgA purification [1,3].

Moreover, for the production of monoclonal antibodies the validation of the purification process is equally important to the purification method. The purification should be able to remove proteins which may cause an adverse reaction in a patient, and the process must demonstrate to remove such contaminants [3].

## 2.3 MONOCLONAL ANTIBODY APPLICATIONS

Monoclonal antibodies have the capability to bind specifically to the antigens to which they are targeted, and hybridoma technology makes the production of these antibodies possible. Uses of monoclonal antibodies include diagnostics, imaging, tissue typing, purifications, treatment of drug toxicity, cancer and autoimmune disease therapy [7,29-31].

### 2.3.1 Diagnostics

The major emphasis of monoclonal antibody use is currently in the diagnostic field. Once characterized, monoclonal antibodies become the most specific reagents for detecting, assaying, and purifying proteins. Monoclonal antibodies are currently being used in drug and hormone assays, tissue and blood typing, tumour antigen identification, and infectious disease diagnosis [7].

### 2.3.2 Tumour imaging

A growing interest exists for the use of antibodies as diagnostic probes in vivo. Radiolabeled antibodies may be used to bind to the surface of cancer cells and so help to locate tumours within the body, which are inaccessible to other diagnostic tests. Also non-malignant lesions in deep tissues can be studied with radiolabeled

Mab's, for instance the delineation of myocardial infarcts using antibodies against cardiac myosin, actin, or enzymes released from damaged muscle [29].

### 2.3.3 Therapy

New research is ongoing for the application of Mab's either alone or bound to radioisotopes, linked to naturally occurring toxins (such as ricin and diphteria), conjugated with cytotoxic agents (such as vinblastine), or as carriers of drug-filled liposomes [7,30]. The human trials using unconjugated Mab-therapy have mostly involved hematological malignant diseases such as leukemias and lymphomas. Studies have demonstrated that leukemia cells can be cleared from the blood by intravenous injections of Mab's. These Mab's exert their activity by coating the cells with antibodies, which are then picked up by the reticuloendothelial system and removed from the circulation [7]. The use of antibody conjugated to radioisotopes, toxins, or drugs, seems to offer greatest hope for the development of cancer-specific cytotoxic agents [29,30]. Monoclonal antibodies may be applicable to a wide range of diseases in which an antibody-mediated process is present, such as the autoimmune diseases myasthenia gravis, systemia lupus erythematosus and rheumatoid arthritis [29,30]. In these disease states, the body generates an immune response against itself. With myasthenia gravis, for example, the body makes antibodies against the acetylcholine-receptor. If a patient's blood was passed through a column of Mab's specific for the acetylcholine- receptor antibody, the antibody could be dialysed out. Another approach of the treatment of this disease is the use of immunoregulation. The T cells, responsible for the regulation of the B cells that produce the antibodies to the acetylcholine receptor, could be destroyed by Mab's conjugates [29]. The uses of Mab's could include the in vivo elimination of drugs in drug toxicities. Mab's targeted to specific drugs, such as digoxin, could be used to treat toxicity caused by toxic ingestions or accumulation because of renal or hepatic failure. A process similar to the dialysis procedure proposed for autoimmune diseases could be used to bind excess drug [7].

### 2.3.4 Development of vaccines

A rather different use of monoclonal antibodies against cellular components is the use of Mab's against parasitic organisms, for example that causing malaria, to purify different antigens from the parasite for use as vaccines [7].

### 2.3.5 Monoclonal antibodies as research tools

Since monoclonal antibodies are so specific for the antigen against which they are developed, they make excellent reagents for purification procedures. For example, immobilized Mab's are used in affinity purification systems of genetically engineered gamma interferon [7].

Monoclonal antibodies will be used for diverse purposes. The clinical use requires a highy purified product. The starting materials, the production method and purification of these complex proteins warrant a detailed quality control demands, which will be discussed in the next chapter.

## 3 QUALITY CONTROL OF MONOCLONAL ANTIBODIES INTENDED FOR USE IN MAN

### 3.1 INTRODUCTION

For the purification of monoclonal antibodies for therapeutic purposes, the method of purification per se, is not as important as the result of the purification process. The purification should remove contaminants which may cause an adverse reaction in patients (proteins, viral and nucleic acid contaminants). As monoclonal antibodies are the product of malignant cells, there is a risk of transferring viruses and nucleic acids associated with malignancy through the product. When the monoclonal antibodies are the products of mice (or other animals), the product should be tested to exclude murine-derived microbial or viral agents. The importance of bacterial toxins such as pyrogens is well recognised: these must also be removed during the purification process and, if the products are for use in vivo, the process should operate aseptically. In developing tests for in-process quality assurance, a manufacturer must bear in mind that the product must comply with regulatory standards. These in-process quality assurance must demonstrate that the product is pure, effective and safe for its intended application.

The different stages of the quality control during the production of monoclonal antibodies will be discussed, starting with the final product.

### 3.2 FINAL PRODUCT

#### 3.2.1 Identity

One of the most important items in the quality control is to prove the identity of the active component in the final product. The most interesting characteristic of a

monoclonal antibody is its specificity [6]. The specificity needs to be established in order to evaluate the usefulness of the antibody. This will be determined to some degree during screening or by using the same antibody assay on different target antigens. There are several assays-immunoassays and biological assays as well as will be outlined below. If an affinity column with the right antigen is available, this would be the simplest method to determine the identity (specificity) of the product. These columns are available [33,34], but up till now these special columns are trade secret and only Registration Authorities have the disposal of the data of the test columns [33,35].

3.2.1.1 Immunological assays

A non-specific antigen assay, which can be used as a screening assay as well as to identify antibody secretion in the bulk harvest, is a binding assay based on Staphylococcus aureus protein A [3]. However, in the mouse IgG2a, IgG2b and IgG3 bind at neutral pH, while IgG1 will bind only weakly at pH>8. In general, mouse IgM, IgA, IgD and IgE do not bind. Very few rat IgG molecules bind protein A [1,25]. Most monoclonals are of the murine IgG-type, so protein A sometimes can be used. Following the non- specific antigen assay, an assay selective for a particular antigen specificity is used.

Solid-phase Radio Immuno Assay (RIA)

Yalow and Berson developed the first radioimmunoassay thirty years ago [36]. The assay is based on the fact that polyvinyl surfaces will thightly adsorb nanograms of most proteins. The antigen is therefore bound to the solid surface and free binding sites saturated with a large excess of an indifferent protein, usually bovine serum albumin. The antibody is then added and left to bind the antigen. Finally, an $^{125}$I-labeled affinity purified anti-antibody or $^{125}$I-labeled staphylococcal protein A is added which detects the presence of antibody bound to the antigen. Radio-activity is measured by a gamma-scintillation counter or by autoradiography using intensifying screens.

Enzyme-Linked Immuno-Sorbent Assays (ELISA's)

The ELISA's are based on a principle similar to the RIA. However, an enzyme, such as peroxidase, alkaline phosphatase or beta- galactosidase is coupled to the anti-antibody instead of the radio-label. Thus the anti-antibody detects the binding of

the desired monoclonal to the surface bound antigen. The amount of desired antibody is then revealed by a color change upon addition of the appropriate substrate [37].

Immunofluorescence screening

It is possible to couple antibodies with fluorochromes (fluorescein, rhodamine) with retention of antigen-binding properties. Cells are then examined for antibody binding using a microscope with appropriate illumination and filters.

A fluorescence activated cell sorter (FACS) may be used to separate those positive populations of cells to which the fluorescein tagged antibody binds [1,10].

Rosetting assays

When an excess of anti-antibodies or protein A-coated sheep erythrocytes are centrifuged with antibody-coated lymphocytes, the erythrocytes will adhere to the surface of the lymphocytes, forming rosettes. Rosetting is a very sensitive and simple method of detection of cell-bound antibody and the only equipment required is a centrifuge and a microscope [1].

Immunoprecipitation and polyacrylamide gel electrophoresis

All supernatants are screened for IgG production by testing their ability to compete with $^{125}$I-IgG for binding to protein A-containing staphylococci. Subsequently, the staphylococci, to which the immune complexes are bound, are harvested and washed. Bound antigen was released by heating the bacteria to 100 °C in SDS-containing sample buffer, and identified by SDS-PAGE and autoradiography [38].

3.2.1.2 Biological assays

Biological assays

Antibodies to an enzyme or hormone might be identified by inhibition of its activity [39,40]. Antibodies which cause passive cutaneous anaphylaxis (PCA) may be identified by subcutaneous injection into rats [41,42].

Cytotoxicity assays

Cytotoxicity assays are used usually only in those occasions where cytotoxic antibodies are of interest. A two-step assay is often used in which the antibody is bound to cells: unbound antibody is washed away, complement is added and cells

are lysed. The release of $^{56}Cr$ (detected by gamma-counting) is typically used as a marker of cell death [1].

### 3.2.2 Purity

Bussard [43] defined pure monoclonal antibodies as an unique family of protein with a defined structure and activity.

The problem of purity could be approached at two levels:

1. the microheterogeneity of proteins produced by one given set of genes and the stability of the structure of the proteins when they are synthesized through many generations.
2. the degree of purity of the preparations of the monoclonal antibodies, meaning free from contaminating proteins, viruses and nucleic acids.

ad 1. Monoclonality can not be proven, it can only be demonstrated beyond reasonable doubt. In practice, one accepts that an antibody is monoclonal when:

a) it is produced by a hybridoma which has been put through a technically satisfactory cloning procedure, or

b)it shows exquisite specificity, which correlates with a preconceived classification. There may still be "silent" immunoglobulin present, but this will not affect the specificity [6].

ad 2. The degree of purity of the preparations: the final monoclonal antibody product should be pure, meaning, free from protein, viral and nucleic acid contamination.

#### 3.2.2.1 Protein

Monoclonal antibody production methods introduce the possibility of protein contaminants. Residual cellular proteins may come from a variety of sources which include media constituents, such as fetal calf serum, cell substrates, and proteins arising from affinity columns used for purification.

Fetal calf serum is usually added to a medium to a final concentration of 10-15%, it contains proteins such as albumin, transferrin, and IgG [4]. To increase the efficiency of myeloma spleen cell hybridization insulin is added to the medium ($10^{-1}$ to $10^{-4}$ IU/ml) [3]. These proteins are a potential source of risk because they may be recognized as antigens by the patient receiving the monoclonal antibody product and stimulate immune responses. They could potentially induce tissue damage by various allergic mechanisms mediated by IgE, IgG, the complement system, and cellular fac-

tors. Bioactive proteins or peptides derived from the cells used in the culture or from contaminating bacteria may be clinically significant.

A large number of continuous cell lines have been found to produce one or more cytokines. These cytokines, e.g. interleukin-2, if present in sufficient quantity could have direct biological effects if a patient receives therapeutic or diagnostic monoclonal antibodies. The problem with many protein contaminants is that they are difficult to measure, in so far as it may not be known exactly which proteins are likely to be present [3]. The Dutch Health Council [44] considered the so-called "biologic response modifiers" (hormones, especially growth factors, and cytokines) as possible risk factors. Under certain circumstances, growth factors can induce proliferation from outside the cell. However, the World Health Organisation (WHO) [45] stated that the theoretical risk that growth factors act as oncogenics is much less than deoxyribonucleic acid (DNA), because: 1. the protein does not have the capacity for self-renewal, and 2. the administered proteins can be active only if they exert their biological effects via the surface of the cell, since the uptake by the cell is so inefficient that no biologically activelevels are achieved. Moreover, they have an extremely short half-life in the blood (e.g. 2 minutes for platelet-derived growth factor) and repeated administration of high concentrations of growth factors (several micrograms per kilogram of body weight daily) would be without oncogenic effect, even in susceptible individuals [45].

Despite the fact that during purification most contaminating proteins are removed [44,46] the Dutch Health Council [44] advises to pay attention to those proteins, their mean arguments advanced are that little is known about the physiology and the effects, particularly at repeated administration.

*SDS-PAGE*

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS- PAGE) is the most widely used method of protein analysis. Electrophoresis is a technique which separates molecules on the basis of their mobility in a electric field. When proteins are heated to 100 °C for 2 to 5 min. in the presence of SDS and reducing agents, they unfold and bind about 1.4 grams SDS per gram of protein. The binding imparts a very strong negative charge to the protein, dominating its native charge. Thus the charge: mass ratio becomes constant for virtually all proteins. Under these conditions, the electrophoretic mobility in acrylamide is inversily proportional to the

logarithm of the molecular weight [47]. Laemmli [48] modified the system, and, nowadays, extremely high resolution is possible.

After electrophoresis the gel is stained with Coomassie blue or silver staining [49]. The latter method is about a 100 fold more sensitive (up to nanogram level) [49].

*Isoelectric focusing*

Isoelectric focusing is an electrophoretic technique in which an electric field sets up a stable pH gradient; proteins move to the unique point where their net charge is zero. This technique is simple and capable of extremely high resolution. Separation is based on isoelectric point-pH at which net charge is zero [1]. However, multiple bands can be obtained with antibodies that are generally regarded as monoclonal [6]. Proteins can be visualized by either Coomassie blue or silver staining as described before.

*High performance liquid chromatography (HPLC)*

As outlined before (2.2.2.3) the intermediate stage of the purification process, adsorption-, ion exchange-, and affinity chromatography are the three techniques used for large scale purification of monoclonal antibodies. Gel filtration, or size-exclusion chromatography, is the technique commonly used for the final purification. To evaluate the production and purification process, SDS-PAGE or two-dimensional gel electrophoresis followed by an appropriate staining method, such as silver staining [49] or Coommassie blue staining, is the usual way of manner [4,23,24].

However, to identify the purity of a product high performance liquid chromatography (HPLC) will be a good alternative.

SDS-PAGE is based on the separation on molecular weight [47]. In the ideal size-exclusion system, molecules are also fractioned on the basis of differential permeability [27]. So the column can be used to differentiate monomeric, dimeric and aggregated forms of Mab's, but also fragments of Mab's and contaminating proteins. Tomono et al. [50] used a TSK-G 3000 SW HPLC column to differentiate monomeric, dimeric, and aggregated forms of IgG. TSK-G 3000 SW is a hydrophilic hard gel, which appears to be suitable for the gelfiltration of proteins by HLPC. The chemical composition of the polymer is unknown [51].

De Rie et al. [46] used the same column to analyze the protein composition. The proteins were detected spectrophotometrically at 280 nm, and the main IgG fraction (99.8%) had a retention time of 9.3 to 9.4 minutes.

Pavlu et al. [23] used three different HPLC techniques, i.e. ion- exchange-, hydrophobic interaction-, and hydroxyapatite chromatography, to purify Mab from ascites fluid. Although the chromatographic patterns (A 280 nm on-line detection) showed sharp peaks, belonging to Mab-activity, the Mab-activity containing fractions on SDS-PAGE showed more than one protein band. This means that there are protein fractions of different molecular present in the sample, due to

(i) fragmented fractions of the Mab

(ii) contaminating proteins

(iii) dimeric or aggregated forms of the Mab.

Ad (i), the fragmentation of the immunoglobulin-molecule can be caused by the reducing conditions of the SDS-PAGE. More research should be done to establish wether HPLC-techniques can replace the SDS-PAGE.

Manil et al. [18] compared gel filtration, anion exchange (DEAE), hydroxylapatite, and affinity (protein A) chromatography. The best results in terms of yield and purity were obtained by the protein A column. However, the peak showed tailing. The major advantage of an affinity column is that the column is able to establish both purity [18] and specificity of the Mab product. However,the elution conditions are rough, due to the antigen-antibody binding forces (see page 13).

To prevent the destruction of the expensive columns milder elution conditions are choosen, which, however, leads to tailing. However, the most important reason that the use of affinity columns is not as widespread as could be expected, is the trade secret of manufacturers producing monoclonal antibodies [33,35].

Combinations of size-exclusion chromatography with affinity-, adsorption- or ion-exhange chromatography are possible to identify and qualify (both quantification and qualificaton) the monoclonal antibody product. More research should be done to determine whether these HPLC techniques can replace the formely used techniques.

3.2.2.2 Viruses

By using mice and rats and also myeloma cells for the production of Mab's, it is possible to introduce viral contaminants. The Dutch Health Council mentions a list of about twenty murine viruses, which should be excluded because of their pathogenic effect [44]. The main concern, however, is the contamination of the final product by retroviruses. The majority of mammalian cells contain in their chromosomes genetic information related to retroviruses. Retroviruses have been shown to be capable of activating or of acquiring oncogenes from cells. They may

also recombine with endogenous leucosis virus to produce a recombinant virus with pathogenic properties [53,54].

During the production process attention should be paid to possible introduction of (non)rodent viruses. The animals used should be healthy and free from infections. Virus particles, however, are often found associated with spleen cells and have been demonstrated in hybridomas. In the original work of Kohler and Milstein Sendai virus was used to promote cell fusion. Now, virtually all fusion protocols use polyethylene glycols (PEG) with nominal molecular weights from 500 to 6,000 [3,6]. It has been reported, that cells are fused in electric fields [3]. In cases where the Sendai virus is used, care should be taken that the virus or its products are not present in the final product. Murine viruses can be detected by the mouse (M) or rat (R) anti-body production (AP) test. The basis of these MAP/RAP tests is the production of antibodies to a virus in a previously infected host. The viral antibodies are tested using tests such as ELISA. However, not every viral infection causes antibodies, e.g. lactic dehydrogenase virus, therefore the LDH enhancement after two days is measured. Thymic virus will be determined in neonatal mice: abnormalities of the thymus are observed after several days [32,56,57]. The rodent and non rodent viruses are detected after inoculation of adult mice, suckling mice, guinea pigs, or embryonated eggs or indicator cell lines, by histological evaluation of the animals or cells at the inoculum site [56,58].

Normal procedures for purification of Mab's are likely to remove or destroy retroviruses [46]. The best approach to separate Mab's from viruses, is affinity chromatography. Molecular sieving or size-exclusion chromatography are also used to separate high molecular weight viruses. Other methods used are:

(i) salt precipitation of Mab followed by filtration through 0.22 μm filters. However, sometimes this method is not stringent enough.

(ii) heat treatment, however, some rodent viruses show extreme heat stability

(iii) some viruses are killed by pH change or by the addition of beta-propiolacton, but these treatments can also reduce the Ig-molecule activity [55].

Retroviruses can be detected by several methods:

1. Electron Microscopy [59]. This semiquantitative method is based on visualizing virus particles by either negative staining with a suspension of latex particles, or sedimentation of virus onto agar blocks, or precipitation of viruses onto gold grids [60], and subsequently direct counting the number of particles per unit area in the electron microscope [59].

2. Reverse transcriptase assay. This method is based on the reverse transcriptase activity of the retroviruses, measured by [$^3$H]-thymidine triphosphate incorporation [61].
3. XC plaque analysis. Murine viruses are growing in media cultures, but do not exhibit cytopathic effects. However, when the XC cell line (a rat tumour cell originally induced by the Prague strain of Rous Sarcoma Virus) is placed in contact with cells infected with murine leukemia virus, syncytium formation occurs. The viruses produce focal areas of infection in tissue culture, which is visualized in lighter staining areas [62].
4. Tritiated uridine labelling. This method is based on [$^3$H]- uridine uptake of growing virus-cultures during a certain time period [63].

A potentially alternative method of making Mab's is by genetic engineering; the technology exists for analysing the composition of the active site of an antibody molecule and for preparing the genetic sequence to code for the site. Such a procedure avoids the use of material that may contain retroviruses [52].

3.2.2.3 Bacteria, mycoplasma and fungi

Bacteria, mycoplasma and fungi are known to have pathological effects on cells. Their presence will limit the yield from hybridomas as well as their conservation [3]. Penicillin and Streptomycin are often added to culture media to avoid bacterial infections [15] in a dose of 100 μg/ml [3]. In emergency situations Kanamycin and Gentamycin can be used, but never in routine, because of the development of resistance of bacteria [15]. A fungal infection is more difficult to avoid, because the useful chemotherapeutica are more toxic to the cells than to the fungi [15]. Moreover, it often leads to the development of resistance and may mask low grades of infection [3]. Fungal infections can be detected by phase contrast microscope [15]. Mycoplasma contamination is particularly troublesome and elimination of the infection is not easy. Many mycoplasmas are small and pliable enough to pass through even a 0.22 μm filter. Goding [1] stated that some random surveys show incidences of infection > 50% in United States laboratories. Primary sources are bovine (fetal calf serum) or porcine (possible from trypsin) and human, and not the tissue of origin of the cells. To reduce the incidence of contamination, fetal calf serum should be heated routinely for one hour at 56 °C and laboratory workers should work aseptically. Identification of a mycoplasma infection may be achieved by microscopic examination of cell culture, as described in the European

Pharmacopoeia [64]. Kaplan et al. [65] have published a procedure based on incorporation of tritiated thymidine. Mycoplasma can also be detected by a test based on hybridization with a 23S ribosomal RNA probe. The probe hybridizes to all mycoplasma species, but not to eukaryotic DNA. The hybridized DNA will stain brightly by the added specific fluorescent dye, Hoechst 33258 [66].

Elimination of mycoplasma is not easy, and should only be attempted if the cell line is particularly valuable. The organisms rapidly acquire resistence to antibiotics. Goding [1] and Peters [15] reviewed the following possibilities to eliminate mycoplasma:

- the use of antibodies
- cultivation with macrophages
- passage through nude mice
- selective DNA breakage with 5-bromo-uracil and visible light
- heating treatment (41 °C, 40 hours) followed by subcloning
- treatment with Kanamycin and Clindamycin followed by recloning.

In general, to fungal, mycoplasmal and bacterial infection alike prevention is preferable to cure.

### 3.2.3 Potency

Zola and Brooks [6] defined the specific activity of the antibody as the reciprocal of the titer per milligram antibody, thus units of activity per milligram antibody. The antibody titer can be determined using the assay selected for screening and determining the highest dilution at which a positive result is obtained. The total protein concentration can be determined by the Biuret method [67], or by the methods described by Lowry et al. [68], or Bradford [69]. The amount of antibody in milligrams can be determined by scanning the gel of (zone)electrophoresis, after staining, with a densitometer, or the HPLC-chromatograph.

Zola and Brooks [6] described the estimation of the immunoglobulin concentration in hybrid culture supernatants using a laser nephelometric method. This method is more precise than the electrophoretic method. However, the results depend on the use of a standard mouse immunoglobulin and an antiserum. Since the Mab contains only one subclass, the apparent concentration will depend on the amount of the anti-antibody in the antiserum, which reacts with the particular subclass, and on the proportion of the subclass in the standard.

It is important to be conscious of the fact that the "unit of activity" is arbitrary and different assays could produce varying results where the way the results are expressed does not assume that the immunoglobulin is all specific antibody. So the potency and purity are together important parameters to evaluate the production and the final product.

### 3.2.4 Sterility

Monoclonal antibody products for in vivo use must be sterile. Monoclonal antibodies cannot be thermally sterilized and therefore must be produced by aseptic processing [3]. This requires the manufacturing process and production facility to be designed to minimize the risk of microbial contamination of the raw materials and final product. Tests for sterility are described in the European Pharmacopoeia [70].

### 3.2.5 Abnormal toxicity

Tests are described in the European Pharmacopoeia [71].

### 3.2.6 Pyrogenicity

Tests are described in the European Pharmacopoeia [72].

## 3.3 THE FINAL BULK MATERIAL

The final bulk, i.e. the finished product, after completion of the manufacturing process, obtained from a bulk harvest of the supernatant or ascitic fluid, should be tested on its purity, activity and its content of adventitious agents.

### 3.3.1 Criteria of purity

The criteria of purity of the final bulk material are the same criteria of purity defined for the final product, meaning that the product is free from contaminating proteins, viruses, and nucleic acids.

The DNA-content of the product is assayed in this stage of the productionprocess.

### 3.3.2 Activity

The activity of the product, meaning the specificity in units per ml product or in units per mg protein as described earlier, is established to confirm that the purification process was successful.

### 3.3.3 Adventitious agents

The final bulk product should be shown to be free from bacterial, fungal, and mycoplasmal contamination and should be tested as described before.

### 3.3.4 DNA content

The final bulk should be tested on its DNA content: the major risk associated with heterogeneous contaminating in biological preparations intended for human use in connected with its potential pathogenic activity. The WHO Study Group on Biologicals [45] concluded that there is negligible risk from heterogeneous contaminating DNA at a concentration of 100 pg or less per parenteral dose.

Experiments have shown that 2 µg of DNA from Polyoma virus or Simian virus 40 can induce tumours in about 50% of animals tested. If 2 µg of DNA is defined as the "tumour-inducing dose" then a residual amount of 100 pg of tumour virus DNA in a biological preparation would correspond to

$100/2x10^{6} = 0.5x10^{-4}$ tumour-inducing dose.

The contaminating DNA, however, normally consists of chromosomal DNA and not of pure viral DNA. If the chromosomal DNA contains an activated oncogene and there is only one copy per genome, the oncogene will represent only 1/10 of the total DNA. Then 100 pg of heterogeneous contaminating chromosomal DNA would contain $100x10^{-6} = 10^{-4}$ pg of activated oncogene, which corresponds to

$10/2x10^{6} = 0.5x10^{-10}$ tumour-inducing dose.

The risk associated with this amount of DNA is so small that it can be safely regarded as being negligible [45]. The Dutch Health Council [44] works also with another calculation. Results of in vitro experiments are extrapolated to the in-vivo situation. In vivo 500 times more DNA than in vitro induces a transformation. Under optimal conditions 1 to 5 µg DNA produces a transformation. Then in vivo 500 to 2500 ug cellular DNA will be necessary. The committee considers 500 µg chromosomal DNA as the "tumour-inducing dose". 100 pg cellular DNA means: $100/500x10^{6} = 2x10^{-7}$ tumour- inducing dose. The American Food and Drug Administration (FDA) [73] allows a maximum of 10 pg DNA per milligram biological preparation.

If maximal 10 to 100 pg of tumour virus DNA is allowed in one milligram biological preparation (protein), a sensitive test is necessary to validate removal of contaminating nucleic acids.

Four methods are available:

1. DAPI complexation [74],
2. Hoechst 33258 complexation [75],
3. Dot blot hybridization technique [76], and
4. DNA spiking [46].



#### 3.3.1.1 DAPI

DAPI, 4,6-diamidino-2-phenylindole 2 HCl, forms a specific complex with DNA, and this fact has been used for quantitative fluorimetric estimation of DNA. The method permits the estimation of DNA concentrations as low as $5x10^{-10}$ g/ml [74]. DAPI is highly specific for adenine-thymidine base pairs [77]. Complex formation depends on the DNA to DAPI ratio, DNA structure, ionic strength, and the presence of essential bivalent anions and cations. Ionic strength of the solutions used should be below 0.1. The fluorescence intensity is highest when DAPI is complexed with native highly polymerized DNA, and decreases with degraded or denatured DNA. Buffers with pH 7 were found to be preferable [74].

#### 3.3.1.2 Hoechst 33258

The method is based on the enhancement of fluorescence seen when 2[2-(4-hydroxy-phenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl)-benzimidazol 3 HCl, usually called Hoechst 33258, binds to DNA. The binding of the compound Hoechst 33258 to DNA is very tight. Labarca and Paigen [75] suggested three possible binding mechanisms:

(i) an intercalation or another form of strong binding that depends on the double-stranded structure and the base composition of DNA, with a preference for adenine-thymidine pairs;

(ii) a strong non-ionic binding, because single-stranded DNA also enhances fluorescence of the compound Hoechst 33258 in a high ionic strenght environment;

(iii) relatively weak binding, because also single-stranded RNA can enhance fluorescence, although to a much lesser extent.

The specificity for DNA is high enough that the method may be used even in crude homogenates [78]. The sensitivity of the assay is 10 ng/ml [75].

#### 3.3.1.3 Dot blot hybridization technique

The method involves immobilization of the nucleic acids on nitrocellulose filters and subsequent hybridization with radiolabeled, nick translated cellular DNA as probe.

Nick translation is an enzymatic reaction in which a piece of DNA is treated with DNase-1 to generate nicks randomly throughout the molecule [76,79]. The nicks are extended using DNA polymerase-1 from E. coli. The DNA is radiolabeled in-vitro by any one of the four [$^{32}$P]-radiolabeled desoxynucleoside-5'-triphosphates. Test samples containing DNA are then denatured by heating or alkali treatment. Small amounts of samples are applied to nitrocellulose filters (dot blotting). Immobilization of the nucleic acids is obtained by heating the filters for 2 hours at 80 °C in a vacuum oven. The homologous, highly radiolabeled DNA probe is also denaturated. The nitrocellulose filters are then incubated with a solution containing the radioactive probe under hybridization conditions. Complementary strands will form stable hybrids, thus binding radioactive DNA to the filter. Radioactivity is determined by autoradiography or by liquid scintillation counting of filter pieces cut into separate dots of sample on the filter. Simultaneous dot blotting of solutions containging known quantities of DNA and of samples to be tested permits a quantitative determination of nucleic acids in the test samples [76]. The sensitivity of the assay is 5 pg DNA [80].

#### 3.3.1.4 DNA spiking

A quite different method of approximation is to add radiolabeled DNA and show that the purification procedure removes DNA. De Rie et al. [46] added [$^{32}$P]-DNA at various stages of the monoclonal antibody purification scheme. The reduction factors are calculated by measuring [$^{32}$P]-DNA before and after every purification step. The total reduction factor was calculated by multiplying individual reduction steps. Instead of a [$^{32}$P]-DNA label, N-acetoxy-N-acetylamino-7-iodofluorene (AAIF) or biotine can be used. By means of an enzymatic reaction, the amounts of DNA are detected by a colorimetric method [81]. Although these tests are very sensitive (1 pg DNA can be detected, with both AAIF and biotine), the [$^{32}$P]-DNA label test has the preference, because it is very difficult to visualize a difference between neighbouring values, especially for the lower values (5 and 10 pg) [81].

### 3.3.5 Discussion

All discussed methods have their pros and cons. The dot blot hybridization technique is the most sensitive assay, but is not totally reproducible [80]. Optimization of the test requires the probes to be basically of the same length as the DNA pieces. Moreover, DNA is only detected on basis of the complement thus a specific piece of DNA is required [80].

The methods using fluorochromes (DAPI [74] or Hoechst 33258 [75,78]) are not sensitive enough to meet the demands of the Dutch Health Council [43], WHO [44] or FDA [73], but are useful to monitor the purification process. The method of De Rie et al. [46] is very sensitive, but no absolute measurements are done, so the originally present amount of DNA should be determined with either of the described methods.

### 3.4 THE PRODUCT

Before the product, meaning the bulk harvest, is purified, it should be tested on its identity, stability and activity [32]. The identity and activity of the hybridoma-product should be established as described before, to decide whether or not further purification is advisable. It is aimless to purify a product with the wrong identity, but it is also useless to purify a product, which cannot be pure at all, because of high costs of purification or which contains contaminating viruses, nucleic acids or proteins, which will not be removed during the purification process [52,56,73,82].

### 3.5 THE HYBRIDOMA

The identity, stability, sterility, and the viral and mycoplasmal content of the hybridoma cell line should be determined [32]. It is essential that the production is based on a clearly delineated seed lot system involving a master cell bank and a manufacturer's working cell bank. These cell banks are homogeneous suspensions of cells, aliquoted into individual containers for storage. The master seed bank is the original cell line on which production is based, which may not necessarily have been produced by the manufacturer. The manufacturer's working cell bank is derived from the master cell bank(s) by a finite passage level [85]. Appropriate precautions should be taken to avoid cross-contamination with other cells. The cell lines should be described in detail, both origin and production data. The characteristics of the hybridoma cell line should be detailed. These include the specificity, class and, when appropriate, subclass of the immunoglobulin secreted, together with any distinguishing features, such as isoenzyme / immunochemical markers. Evidence that the cells of the lot are free from microbial contamination (viral, bacterial, mycotic, mycoplasmal) should be provided. The proposed purification procedure used in the production of the monoclonal antibody should be shown to be capable of removing and/or inactivating the contamination. If not, it should be considered to choose

another purification process or to destroy the hybridoma cell line. Information should be provided on the stability of antibody secretion by cells of the cell banks after storage and recovery. Samples of the seed must be retained until at least after the expiry date of the resulting final lot [52,56,73,82].

### 3.6 MYELOMA X SPLEEN

The source material, the myeloma cells and the immune parental cell, should be described in detail. The source, name and characteristics of the cells should be given. The health of the donor(s) should be provided. Any relevant clinical data on the donor must be reported and cryopreserved samples of the cells should be retained in case retrospective investigations become necessary. The fusion, cloning, and recloning should be described. The area and the work of the staff should be aseptic to avoid contamination [32,52,56,73,82].

## 4 QUALITY CONTROL OF MEDICINAL PRODUCTS DERIVED FROM RECOMBINANT DNA TECHNOLOGY

### 4.1 INTRODUCTION

The principle of recombinant DNA (rDNA) technology is that genetic information is isolated from a donor organism, and is cleaved by restriction enzymes (endonucleases) into individual pieces. These pieces are incorporated in a carrier DNA and transferred with this vector into another cell. The best known cloning vehicle is probably the bacterial plasmid pBR 322, this vector was created artifically by using different parts of certain naturally occurring plasmids. Subsequently, the cells which harbor the rDNA molecule containing the gene of interest are determined. The desired clones can be selected on the basis of the presence of the vector or of the inserted gene itself. For example, some plasmid vectors confer resistance to an antibiotic. In Escherichia coli (E. coli) pBR322 confers resistance against ampicillin and tetracyclin. The restriction enzymes cuts in the tetracyclin resistance region. Subsequently, with the DNA ligase enzym a foreign DNA is build into the vector and the hybrid has resistance to ampicillin only. Another approach is to determine which cells bind RNA that is complementary to the gene of interest or synthesize protein encoded by it [83,84]. The choosen clones are cultured (fermentation) and after harvesting the desired product is purified by techniques normally used for protein purification [83].

Just as for monoclonal antibody production, the in-process controls for recombinant DNA are very important, because the final product, normally a complex protein, eventually glycosilated or linked with disulfide bonds, is not easy to analyse when compared to a conventionally produced new chemical product [85,86].

Moreover, the ability to synthesize and manipulate nucleic acids allows the construction of genes coding for modified products possessing enhanced biological activity and/or diminished undesirable characteristics, or entirely new products [86].

The choice of manufacturing procedure may influence the nature and range of potential contaminants. Thus rDNA-derived products may contain potentially hazardous contaminants not normally present in their equivalents prepared by conventional methods and for which the purification processes must be shown capable of removing. Examples of these are endotoxins in products expressed in bacterial cells and DNA of oncogenic potential in products expressed in transformed mammalian cells [85,86]. Another factor compromising the safety and efficiency of rDNA-derived products is the unintended variability in the culture during production which may lead to changes which favour the expression of other genes in the host/vector system or which cause alterations in the polypeptide product. Such variation might result in decreased yield of the product and/or quantitative differences in the impurities present in the product [85,86].

Both authorities [85-88] and manufacturers [90,91,98] are employing guidelines and points to consider on the production and quality control of (medicinal) products derived from recombinant DNA technology.

The requirements [32] should be generally applicable, individual products may present particular quality control problems. Thus the production and control of each product must be given careful individual consideration taking fully account any special features. The aim of this part of the paper is to give a review of general points of the quality control of rDNA products. Some products will be discussed more detailed.

## 4.2 FINAL PRODUCT

### 4.2.1 Identity

Rigorous characterizatiom of the active substance by chemical and biological methods will be essential. Routine detailed characterization of the final product may be required if the nature of the expression system makes it impossible to characterize the gene at the production level. Physico-chemical properties of the

molecule, for instance size, charge, isoelectric point, amino acid composition and hydrophobicity can be determined by techniques normally used for protein analysis such as polyacrylamide gel electrophoresis (PAGE), isoelectric focusing, size exclusion, reversed phase-, ion exchange-, hydrophobic interaction- or affinity chromatography, peptide mapping, amino acid analysis, light scattering, UV spectroscopy, circular dichroism (CD) and optical rotary dispersion [88] and other spectroscopic techniques [86]. It may be desirable to include suitable tests to establish that the product has the desired conformational structure and state of aggregation. For this purpose techniques a two-dimensional (2D) SDS-PAGE and CD are advised [88]. Janssen [92] suggested fast atomic bombarding-mass spectrometry (FAB-MS) for determining the structural analysis of the proteins. This technique is based on splicing a large polypeptide in smaller peptides by an atomic bombardment. The peptides are analysed by mass-spectrometric techniques. The advantages of this technique over 2D-SDS-PAGE or CD are the reproducibility (splicing occurs always at the same place), and the unambiguousness of the results. Especially, the CD-results are hard to interpret [92].

Immunological methods such as RIA and ELISA can be used to identify the protein as well as to establish the potency of the batch [88].

Depending on the extent of other identificaton tests, sequence verification of a number of amino acids at the N- and C-terminus or other methods such as peptide mapping should be performed [85,86,88], because these termina are the points where modifications or alterations in the polypeptide product appear quickly and easily [92]. Attention should be paid to the possible presence of N-terminal methionine, signal or leader sequences and other possible N- and C-terminal modifications (for instance acetylation, amidation or partial degradation by exopeptidases). Other post-translational modifications, such as glycosilation should be indicated [86]. Only eukaryotic cells (e.g. yeast, mammalian cells, are able to add glycomoieties to the protein backbone after or during its synthesis on ribosomes. The nature of the carbohydrate added to the protein is dependent on the host cell. This can lead to unwanted changes in the biological properties [91], which should be examined.

Pestka [93] reviewed some techniques for the amino acid analysis. High performance liquid chromatography is the mainstay of current isolation methods, but the trend in the 1990s will be to integrate electrophoretic methods into purification and structural analysis.

Beside the full characterization of the active component, attention should be paid to the formulation: buffers, stabilisers and any other additives [86]. Also the colour, clarity and pH shall be outlined.

### 4.2.2 Purity

The degree of purity and also the degree of consistency of the production process are important items falling under the head "purity". In general, a very high degree of purity can be achieved for most products by modern manufacturing procedures. Dependent on the nature and intended use of the product one or more purification steps as described before are choosen.

Just as discussed under 'purity' of Mab's, the final rDNA technology products should be pure, meaning, free from contaminating proteins, viruses and nucleic acids. Five groups of contaminants may be present in the final product(s):

1. contaminants arising from the host cell (e.g. proteins, nucleic acid and carbohydrates)
2. those coming from the culture medium (e.g.antibiotics)
3. the products of extraneous contaminants (bacteria, viruses, fungi and moulds)
4. endotoxins
5. dimers, polymers and degradation products [S. Vargo, 91].

#### 4.2.2.1 Contaminating proteins

In consequence of the production process and purification procedures, contaminating proteins are introduced. Unintended variability in the culture during production may lead to changes in the host/vector system which may cause alterations in the polypeptide product. Consequently, procedures to ensure consistency of production conditions as well as the final product are necessary. The choice of manufacturing procedure may influence the nature and range of potential contaminants. Residual proteins may come from a variety of sources, including media constituents (fetal calf serum), bacterial cells, in which the product is expressed, and special attention should be paid to the affinity chromatography columns in case monoclonal antibodies are used. The purity of each batch should be established and be within specified limits. Analytical techniques used are SDS-PAGE, isoelectric focusing and chromatographical methods [85,88].

#### 4.2.2.2 Viruses

Viruses can commonly contaminate the animal species from which the cell line has been derived. Certain cell lines contain endogeneous viruses, e.g. retroviruses, which may not readily be eliminated. The presence of these organisms, under a variety of conditions known to cause their induction, should be tested as described before. Furthermore, the purification procedure should be shown to be capable of removing and/or inactivating any such virus which may be inevitably be present in the seed as an endogenous agent or part of the expression vector [85].

#### 4.2.2.3 Bacteria, fungi and mycoplasma

Bacteria, fungi and mycoplasma can be, even as some viruses, infective adventitious agents and must therefore be prohibited.

#### 4.2.2.4 DNA

The analysis of the final product, but also the products at the different stages of the production and purification procedure should be tested on DNA-content, as described earlier. In order to remove DNA, RNA and fragments of these substances, it may be necessary, besides the purification procedure to treat the pooled ascites or tissue culture fluids first enzymatically with endonucleases [88].

#### 4.2.2.5 Culture components

If antibiotics, antimicrobial preservatives or additives have been used, either in the production, concentration or purification, their concentration shall be assayed [88].
Penicillin and other beta-lactam antibiotics shall not be used. The culture media shall be free from ingredients that will be present in the final product and that are known to cause toxic or allergic reactions in man [86,88].

#### 4.2.2.6 Auxiliary agents

Information about buffers, stabilisers and any other additive should be in accordance with the European Pharmacopoeia [89].

### 4.2.3 Potency

The potency of each batch of the product should be established (e.g. units of biological activity per ml) using, wherever possible, an appropriate national or international reference preparation calibrated in units of biological activity [85,88]. It

is recommended to standardize (as soon as possible) the units of measurements, to prevent mistakes [94]. As long as an international standard preparation is not available a part of the lot that has been proved to be effective in field trials may be used as a standard preparation [86,88]. In addition, information on specific activity, i.e. units of (biological) activity per unit weight of product, will be of considerable value and should be reported too [85,86].

Meager et al. [95] reviewed assays for tumour necrosis factor (TNF) and related proteins. In addition, their conclusions, derived for one specific rDNA product can be used to other rDNA-derived products. Assays can be divided in bioassays and non-biological assays.

#### 4.2.3.1 Bioassays

Bioassays can be subdivided in tests done in vivo and tests done in vitro [96].

The original assay giving TNF its name was based on in vivo tumour necrosis. Fibrosarcoma was implanted in a mouse. After seven days, the factor was administered and necrosis was scored on a graded response 24 hours later. More in-vivo assays are desribed [96], but in general, the in-vivo assays require large amounts of material and are semiquantitative in nature. The need for more sensitive assays has stimulated development of several in-vitro assays [95].

The in-vitro bioassays to determine TNF are mostly based on the cytotoxic effects of TNF on cells, but the suppressing of lipoprotein lipase activity of TNF (cachectin) can also be the basis of a test. Disadvantages of in vivo tests are that the observed effects can be false positive or negative due to a multitude of endogenous and environmental factors. For instance interleukin-1 (Il-1) is an important factor disturbing the assay, because Il-1 shares several biological activities with TNF and can itself be cytocidal to some tumour cell types [95].

#### 4.2.3.2 Non-biological assays

The lack of specificity of biological assays has generated great interest in the development of non-biological assays, especially immunoassays, for their quantification. The major advantage of immunoassays (IRMA, ELISA) over bioassays is that they are truly specific for the desired protein. Other factors favouring the use of immunoassays are:

(i) a high degree of precision and reproducibility

(ii) shorter operation period, and

(iii) insensitivity to the modulators of the protein.

The major disadvantage is that while the immunoassays are capable of detecting biological active proteins, it is impossible to guarantee that the assay will not also recognise inactive denatured, aggregated or fragmentated molecules [95]. So complementary tests, such as size exclusion chromatography are necessary.

Another disadvantage is that some immunoassays are so specific, that they require to be calibrated with standard preparation containing the precise protein activity to be measured. For instance glycosylated or non-glycosylated species will have different standards [95].

It is recommended that correlations between potency measurements, involving biological tests, and the results of physico-chemical methods are made and that this information is reported. If possible, batches should be calibrated using accurate physico-chemical tests, and the biological assays used to confirm - within stated limits-that the product is biological potent.

### 4.2.4 Sterility

Tests for sterility are described in the European Pharmacopoeia [70].

### 4.2.5 Abnormal toxicity

The Dutch National Control Laboratory [86] stated that each final bulk should be tested for abnormal toxicity according to the method described in the European Pharmacopoeia [71]. However, knowing that most natural substances and chemicals of high molecular weight are antigenic, inducing replication of specifically primed lymphocytes and usually leading to the formation of antibodies, more attention should be paid to this subject. Although contaminating proteins, viruses and nucleic acids, etc. cause adverse reactions and can be remarked as toxic, they are not the subject of discussion in this chapter. Bass and Scheibner [97] suit for toxicological experimentation and guidance, which are needed both for the safety of the patient and the registerability of the drug. They described acute and chronic reactions due to the administration of biotechnological products in humans or animals. The cause and the impact of toxicity described is hard to interpret, but seem clear.

Bass and Schneiber [97] point out a structural approach to toxicity testing:

1. performance of safety pharmacology studies. These studies should be performed with all products at the beginning of toxicological investigations. Both pharmacological active and higher doses should be tested in several species from rodents to primates, to determine the most sensitive.

2. performance of acute toxicity and mutagenicity studies
3. detailed analysis of safety pharmacology in short-term experiments. These studies determine direct toxic reactions and effects secondary to therapeutic effects.
4. controversial studies - monitoring of antibody formation.

Very important of those studies is the selection of species. The European Federation of Pharmaceutical Industries' Associations (EFPIA) stated that the classical toxicological tests are of limited value, because most rDNA-derived proteins are species-specific and thus immune-reponse can not be predicted [89]. It is therefore important to perform toxicity tests in primates.

In principle species-specific proteins may exert toxic effects on the progeny [90]. Problems with pregnancy and malformations can be caused by both contaminants (viruses, toxins, pyrogens) and the purified protein (immunogenicity). Herewith, it should be taken into account that the substance does not necessarily cross the placental barrier to exclude reproductive-toxicological risk [99].

It is difficult to say something in general about toxicity, mutagenicity and teratogenicity, so it is to be recommended to study every compound on a case-by-case basis.

#### 4.2.6 Pyrogenicity

Tests for pyrogenicity are described in the European Pharmacopoeia [72].

### 4.3 FINAL BULK

Purity, potency, sterility, abnormal toxicity and pyrogenicity tests are performed on the final product. In addition, the final bulk processed product, meaning the finished product, after completion of the manufacturing process, obtained from a bulk harvest, is tested on the presence of rodent and non-rodent viruses, mycoplasma and DNA-content. For description of these tests see chapter 3.2.2.2 and 3.2.2.3 respectively.

### 4.4 PRODUCT

Before purifying the desired protein, the product of the cell line is tested on its identity, stability and activity. Rigorous characterization of the product by chemical and biological methods will be essential. The stability of the cell line should be proved, the yield does not vary beyond defined limits and the nature and quality of the product does not change with respect to specified parameters. The identity of the

product is characterised by tests as described before: the methods employed should include tests for anticipated biological activity as well as physicochemical and immunochemical methods. At the same time the purity of the product will be studied in relation to the strategy for harvesting and purification. Criteria for rejection of harvests and premature termination of the culture should be defined [86].

### 4.5 ENGINERED CELL LINE

It is essential that production is based on a well defined seed lot system involving a master seed and manufacturer's working seed bank.

A master seed bank is a homogeneous suspension of the original cells already transformed by the expression vector containing the desired gene, aliquoted into individual containers for storage (e.g. in a liquid nitrogen refrigerator) [86,88].
In some cases it may be necessary to establish separate master seed banks for the expression vector and the host cells.

A manufacturer's working seed bank is a homogeneous suspension of the seed material derived from the master seed bank(s) by a finite passage level, aliquoted into individual containers for storage (also in a liquid nitrogen refrigerator, by example) [85,86]. In both seed banks, all containers are treated identically during storage, and once removed from storage, the containers are not returned to the seed stock. A critical part of quality control will involve the full characterization of seed material. Where higher eukaryotic cells (e.g. mammalian origin) are used for the production, distinguishing markers of the cell, such as specific isoenzyme and immunological features or karyology, will be useful in establishing the identity of the seed. Details of the tumourigenicity of continuous cell lines should also be obtained and reported. Likewise, where microbial cultures (e.g. E. coli) are used, specific phenotypic features which form the basis for identification should be described [85,86,88,98].

#### 4.5.1 Viruses, mycoplasma, sterility

Evidence that the seed lot is free from potentially oncogenic, where appropriate, or infective adventitious agents (viral, bacterial, fungal or mycoplasmal) must be provided [85,88]. Special attention should be given to viruses which can commonly contaminate the animal species from which the cell line has been derived [56]. Certain cell lines contain endogenous viruses, e.g. retroviruses, which may not readily be eliminated. The purification process should be shown to be capable of re-

moving and/or inactivating any such virus which may inevitably be present in the seed as an endogenous agent or part of the expression vector [85]. In case of a mammalian host cell, the parent seed should be free from reverse transcriptase and free from virus-like particles, shown by electron microscopy [88].

### 4.5.2 Production

If possible not more than one cell line should be cultivated simultaneously in the same production area. If not, and other cell lines are cultivated in parallel, special care should be taken to prevent cross-contamination [85]. Production areas shall be decontaminated before they are used for the manufacture. The production shall be conducted by staff who have not handled other infectious micro-organisms or animals in the same working day. The staff shall consist of persons who shall be examined medically and found to be healthy. Persons not directly concerned with the production processes, other than official representatives of the National Control Authority, shall not be permitted to enter the production area [88].

There are two possible production strategies:

1. production at finite passage - this cultivation method is defined by a limited number of passages or population doublings, which must not be exceeded during production [85].
2. continuous culture production - the number of passages or population doublings is not restricted from the beginning of production. Criteria for the termination of production have to be defined by the manufacturer [85].

In case of production at finite passage, for each production run details of the fermentation or culture used should be established. When is choosen for a continuous culture production, it is necessary to monitor the production system throughout the life of the culture. Yield, nature and quality of the product are important parameters to establish the stability of the system, because of possible chromosomal abnormalities, which can lead to tumourigenic changes [88].

Both production systems require regulator tests for microbial contamination [85,88].

### 4.5.3 Expression system

The manufacturer shall provide a description of the method used to prepare the segment coding for the desired product, including both the cell type and origin of the source material, a detailed nucleotide sequence analysis and a restriction enzyme digestion map of the cloned segment. If a cloned polynucleotide contains more

information than the coding sequence, i.e. introns flanking sequences, then these additional sequences shall be identified. The construction of the vector used for expression of the cloned nucleotide segment into its respective product shall also be thoroughly described. This description shall include a detailed explanation of the source and function of the component parts of the vector, e.g. origins of replication, antibiotic resistance genes, promotors, enhancers, whether or not the product is being synthesized as a fusion protein. The restriction enzyme digestion map of the entire constructed vector shall be provided [85,88,98].

#### 4.5.4 Host cell

A complete description of source, phenotype and genotype of the host cell shall be provided [85,88]. In the case of cells of mammalian origin, data concerning its tumourigenicity should be determined [98]. The mechanisms of transfer of the expression vector, copy number and the physical state of the vector inside the host cell, integrated or episomal, shall be provided. The measures used to promote and control the expression system shall be described in detail [85,88,98].

Mammalian cell lines may have significant advantages as expression systems for some types of gene products cloned by recombinant DNA methods. These advantages are the higher yields for some products, glycosylation of products, ability to fold nascent polypeptides on endoplasmatic reticular membranes, the secretion of products completely outside the boundaries of the cell, and the optimal preservation of product quality. Disadvantages of these cell lines are that more medium and, moreover, an extremely long production period are required [99].

## 5 BIOLOGICAL RESPONSE MODIFIERS

### 5.1 INTRODUCTION

Biological response modifiers (BRM), also called biomodulators or "biomodulins", are agents and approaches whose mechanism of action involve the individual's own biological response. Biological response modifiers can act in several ways:

1. Increase the host's defenses by administering natural biologicals or the synthetic derivatives thereoff as effectors or mediators of an antitumour response,
2. Increase the individual's antitumour responses through augumentation and/or restoration of effector mechanisms, and/or decrease a component of the host's reaction that may be deleterious,

3. Augment of the individual's responses to modified tumour cells or vaccines, which might stimulate a greater response by the individual or increase tumour cell sensitivity to an existing response, e.g. by changing the characteristics of the membrane of tumour cells or making the tumour cells more susceptible to killing by immunological mechanisms or cytoxic drugs,
4. Decrease the transformation and/or increase differentiation (maturation) of tumour cells,
5. Increase the ability of the host to tolerate damages by cytotoxic modalities of cancer treatment [100,101].

The prototype of BRM's are Bacille Calmette Guerin (BCG), an attenuated strain of Mycobacterium tuberculosis, and Corynebacterium parvum. Both agents affect several components of the immune response, modify the activity of several cell types and may induce positive (stimulatory) or negative (inhibitory) effects depending on the system and how it is used. However, their anti-tumour effects are limited [2].

The different BRM's are proteins and are often grouped into families. There is considerable overlap in the activities of the factors, however; structurally unrelated molecules, such as TNF and interleukin-1, elicit many of the same effects [100]. The different groups will be discussed. The production, characterization and quality control will be underlined, but also the mechanisms of action and clinical potentials will be shotrly reviewed.

Advances in molecular biology, and especially recombinant DNA and hybridoma technologies have made it practical to produce massively purified BRM's for clinical application.

## 5.2 TUMOUR NECROSIS FACTOR AND LYMPHOTOXIN

The Russian doctor and writer Anton Chekhov M.D. wrote to Alexei Suvorin on December 24, 1890 in Moscow the following letter:

"Cancer is not a microbe; it is tissue growing in the wrong place, which, like a weed, chokes all the tissues in its vicinity. If Hay's uncle shows improvement, it would be merely because the erysipelas germ, i.e. the elements producing the disease of erysipelas are also elements of the Kochini. It has long been noted that the growth of malignant tumours halts for a time when this disease is present." (translation Gressner [103]).

The description of spontaneous regressions of some human tumours by infecting the cancer patients with live bacteria is usually ascribed to William B. Coley (1894).

However, the results were inconsistent, and radiation therapy and chemotherapy essentially supplanted Coley's approach. His daughter, Helen Coley Nauts, continued his work in the Cancer Research Institute. These investigators confirmed that a range of infection agents and their products had anti-cancer effects in animals. In particular, they demonstrated that the injection of live or killed strains of gram-negative bacteria could cause hemorrhagic necrosis of mouse tumours: the tumours bled into themselves, turned black and dried up [104]. Muray J. Shear et al. identified and purified the active component of the gram-negative bacteria, determining that it was a complex fat-and-sugar compound now called lipopolysaccharide [102]. Baruj Benacerraf and Lloyd J. Old discovered that Bacillus Calmette-Guerin plays also an important role in the destruction of cancer [102]. In 1975 Carswell et al. [104] described that both bacterial endotoxins and BCG caused tumour necrosis. Mannel et al. (1975) and Matthews (1980) proved that the TNF is produced by macrophages [105] and monocytes [106]. In 1985 Aggarwal et al. [107] described the production and characterization of human TNF. Recent studies [108] suggest that TNF is identical with cachectin, a macrophage hormone, originally isolated in the course of studies aimed at delineating basic mechanisms of cachexia in chronic disease [96,102,109,110].

### 5.2.1 Mechanism of action

TNF is a cytokine, which plays a central role in immunological processes. It is important by the immunological reaction against virusses (inhibition of virus replication) and the reaction against tumours (in vitro it appears to be toxical to some tumour cell lines).

The post-receptor effects of TNF are only known in general terms [109,110]. The protein acts to suppress biosynthesis of several adipocytes-specific mRNA molecules and to prevent morphologic differentiation of pre-adipocytes. Presumably, lipoprotein lipase is one of many enzymes specifically suppressed at a transcriptional level by the action of this hormone. TNF also induces the biosynthesis and/or release of specific proteins, including Class I Major Histocompatibility Antigen, Granulocyte-Macrophage Colony-Stimulating-Factor and Interleukin-1 [102].

TNF is said to act by diminishing tissue perfusion by mechanical obstruction of regional blood flow. The protein alters the hemostatic properties of the vascular endothelium, inducing the production of a procoagulant activity, and inhibiting the expression of thrombomodulin at the cell surface. This causes accretion of thrombi,

leading to disseminated intravascular coagulation at a systemic level and to occlusion of tumour vessels [109,110]. TNF has also a direct toxic effect on vascular endothelial cells, by inducing the interleukin-1, leucotrienes and platelet-activating-factor production. By inducing the release of interleukin-1, TNF causes fever, hypotension, neutropenia and thrombocytopenia. Rats injected with TNF died because of dyspnoea, the lungs showed oedema, and both the intestine and kidneys were necrotic [109,110].

The protein has also an effect on the transmembrane potential of muscle cells: the sodium permeabilization is not compensated, so the natrium-kalium ATPase is inefficient [110]. Like interleukin-1, TNF is an osteoclast activating factor, and it also stimulates the production of prostaglandin E and collagenase.

Taverne et al. [111] found that TNF could kill malarial parasites in vitro and in vivo.

More research is needed to prove if the tumour-killing and the other effects will be of clinical/therapeutical significance.

### 5.2.2 Production and purification of TNF

Aggarwal et al. [107] described the production, purification, and characterization of TNF in 1985, just a few months before Beutler et al. [112] published the production and purification of cachectin. In the same year, Beutler et al. [108] proved that both fators possess the same biological activities in vitro, but also the amino acid composition reveal strong similarities and both proteins are acidic and hydrophobic, containing identical numbers of threonine, valine, alanine, histidine, arginine and cysteine residues. These findings suggest that TNF and cachectin are the same proteins. Human TNF has been cloned and expressed in high yield in E. coli and bacterial expression now accounts for virtually all the TNF used clinically [109].

### 5.2.3 Lymphotoxin

Lymphotoxin (LT), renamed by Shalabey et al. [115] as TNF-β , is produced by mitogen-activated lymphocytes and was initially identified as a biological activity with anti-cellular effect on several tumour cell lines. Some neoplastic cell lines are directly lysed by LT, while others are growth-inhibited. Primary cell cultures and cell lines are not growth-inhibited [116,117]. LT is said to act synergistically with type I interferons [116]. It has been suggested that LT and TNF are acting on the same receptor, because they exhibit the same biological activities [117]. Cloning of

LT [116] and TNF [114] complementary DNA showed that both biological activities and chemical structure are closely related.

### 5.2.4 Molecular characterization

TNF and LT have 36% of their amino acids in common; this homology increase to 51% when conservative replacements of amino acids are taken into account [114,116].

### 5.2.5 Assays

The quantification of TNF activity depends mostly on biological assays. Both in vivo and in vitro assays have been developed by researchers in the field. Except these bioassays, alternative, non-biological assays, such as immunoassays, have been developed [118,119]. Up till now no international standard for TNF or LT has been established, so national authorities (such as the National Institute for Biological Standards and Control, NIBSC) have made available interim standards for TNF which have been formulated from purified recombinant human TNF [95]. Important is the fact that the results of the immunoassays can be dependent on the formulation of the production process [95].

## 5.3 INTERFERONS

### 5.3.1 Introduction

Interferon (IFN) was discovered in 1957 by Isaacs and Lindenmann [120] as of its very potent antiviral mechanism. For several years it was regarded only as the main mediator of viral interference (that's why it is called interferon, from "to interfere"), although it soon became clear that other cell functions were affected as well. In 1980, an international group of scientists attempted to devise a system to define and classify the interferons. The committee accepted the definition for interferon: "To qualify as an interferon a factor must be a protein which exerts virus-non-specific, antiviral activity at least in homologous cells through cellular metabolic processes involving synthesis of both RNA and protein." [121].

Interferons will be classified into types on the basis of antigenic specificities,type designations to be α, β and γ corresponding to previous designations of leukocyte, fibroblast, and immune or type II interferons, respectively.

α- and β-IFN's are usually acid-stable and correspond to what have been called type I IFN's; γ-interferons are usually acid-labile and correspond to what has been called type II IFN's [121].

### 5.3.2 Mechanisms of action

In addition to their viral activity, the interferons have other activities, including inhibition of cell growth, antitumour action, effects on cellular differentiation and a wide range of immunomodulatory actions. The immunomodulatory effects of IFN's are particularly important [122]. Interferons interact with cells through specific cell surface receptors. The family of α-interferons and β-interferon bind to a common IFN-type I receptor, whereas γ-interferon binds to a different receptor, the IFN-type II receptor. Langer and Pestka [123] detailed the different characteristics of these receptors.

α-interferons are produced by lymphoid cells. They are not induced only by viruses, but also by xenogenic or tumour cells, bacteria and B cell mitogens.

β-interferon is produced by fibroblast and epithelial cells during viral infections. The induction requires virus internalization and exposure of nucleic acid. Because of its poor diffusion from solid tissues, β-interferon can only control viral replication and spreading effectively at the site of the infection.

γ-interferon is produced by lymphocytes stimulated by specific antigens or by T cell mitogens; the presence of macrophages is usually required for optimal production.

Cells respond to IFN through activation and expression of specific cell genes, that occur at different times for the three types of IFN's. α- and β-INF induce directly the effector (antiviral) molecules by transcription and translation of new products. This occurs in a one-step derepressional process, which takes no more than 30 minutes for production of specific mRNA and no more than 1 hour to establish the antiviral state.

γ-INF acts via a multi-step derepressional process, and is slow (several hours instead of a few minutes) [122,123]. Virtually all phases of the virus growth cycle have been shown to be affected by IFN, at least at some extent [122].

The effect of IFN's on tumour growth is still not fully understood. Two main mechanisms may be involved:

(i) a direct inhibition of tumour cell division,

(ii) activation of immunocompetent cells.

Tumour growth might be affected also by other, still scarcely defined mechanisms, such as modulation of cell membrane antigens and hormone-like activity [123].

### 5.3.3 Therapeutic applications

IFN's are possibly effective in virus infections, particularly by some herpes infections (keratitis, herpes labialis, herpes genitalis) and rhino- or influenza virus, when administered intranasally. Some solid tumours and some haematologic malignancies have decreased in measurable tumour mass or in abnormal proteins, after administering IFN's [124-126].

However, much more research should be done to establish the role of IFN as therapeutic agent. Special attention should be paid to side effects, pharmacokinetics, and efficiency. Recombinant DNA technology maked it possible to produce IFN's in large amounts, so clinical trials with application of IFN as tumour agent are being performed and registration of recombinant IFN followed.

### 5.3.4 Characteristics of the interferons

Interferons are acting species-specific, not virus-specific [125].

## 5.4 INTERLEUKINS

Interleukins (Il's) were defined at the Second International Workshop in Lymphokines as soluble factors, produced by and acting on leucocytes [127]. Interleukins belong to the group of "lymphokines", which are non-antibody proteins or glycoproteins generated by lymphocyte activation that act as intercellular mediators of the immunological response. The International Union of Immunological Societies is engaged on the nomenclature of, among others, the lymphokines. Up till now, several interleukins have been described [128-130,132,134-139,142-147,150].

## 5.5 COLONY STIMULATING FACTORS

### 5.5.1 Introduction

Colony stimulating factors (CSF's) are a group of proteins which stimulate the in vitro growth and colony formation of granulocytes and macrophages from progenitor cells found in bone marrow. Colony-stimulating activities have been identified in various body fluids, tissue extracts, and media conditioned by certain cell cultures.
To date, four subclasses of CSF's have been described:

| | |
|---|---|
| M-CSF or CSF-1: | stimulates the formation of macrophage-containing colonies |
| GM-CSF: | stimulates the colony formation of both neutrophilic granulocytes and macrophages |
| G-CSF: | stimulates neutrophilic granulocytes |

multi-CSF: stimulates erythroid granulocyte and macrophage colonies, usually called interleukin 3 [141,152,153].

In vivo, these factors are necessary for hematopoietic proliferation and differentiation. They are relatively specific in their actions and in the distribution of their receptors, but to their ranks must be added several other factors that can, directly or indirectly, influence colony formation. For instance, interleukin-1 acts on highly purified stems to synergize with GM-CSF or M-CSF in stimulating colony formation. The radioprotective effects of Il-1 are thought to be due to the stimulation of stem cells to a point in the cell cycle where they are more radio-resistent. Il-1 may be effective in conjunction with other CSF's in protection of myeloid function against insults such as radio- or chemotherapy [141].

### 5.5.2 Clinical uses of CSF's

In patients receiving autologous bone marrow transplants for various forms of leukemia, GM-CSF accelerated the rate of recovery of circulating granulocytes compared with historical controls. G-CSF was administered to patients undergoing chemotherapy for solid tumours in which there was no hematopoietic involvement, and could shorten or prevent the period of neutropenia associated with chemotherapy.

Patients with myelodysplastic syndrome have been treated with GM-CSF, a dose-dependent increase in white blood cell counts was observed [141].

In summary, overviewing the facts, CSF's will be used in fighting hematological dysfunction in a number of diseases.

### 5.5.3 Biochemical characterization

The CSF-induced proliferation of bone marrow can be monitored by three different assays:

(i) colony formation of cells in liquid culture,
(ii) cluster formation in 0.3% agar, and
(iii) incorporation of [$^3$H]-thymidine.

The activity is described as the colony forming units per mg product [153].

## 6 GENERAL DISCUSSION

Monoclonal antibodies as well as recombinant DNA technology products are complex proteins, sometimes glycosilated or linked with disulphide bonds, which are

not easy to analyse when compared to a conventionally produced new chemical product. Therefore, the in-process control appears to be an integral part of the quality control of these substances.

The quality control extends over three steps of production:

- the first step deals with the "pure" biotechnological part of the active ingredient production up until a hybridoma or a cell line,
- the second step deals with the biotechnological-pharmaceutical part of active ingredient production, meaning production and purification of the Mab's or the rDNA technology products,
- the third step is a pharmaceutical phase of production and begins when the active ingredient is delivered for pharmaceutical production.

Because the different steps of the production process are closely related, and especially the final product-control cannot be separated of the in-process controls, it is important for pharmacists (who are concerned with quality control) to speak the language of biotechnologists, biologists and biochemists. In this paper, a review is given about the theory of monoclonal antibodies and the recombinant DNA technology, and the consequent quality tests.

The main problems are inherent to the group of products, because of:

- the origin of the material: contaminating proteins from the host cell, tumourigenic material, animals containing viruses;
- the production process: culture medium containing antibiotics, introduction of viruses and bacteria;
- the purification process: the formation of dimers, polymers and degradation products, and contaminating proteins from columns.

At the same time one should consider the extraneous contamination of bacteria, viruses, mycoplasma, fungi, and endotoxins.

Several tests have been developed to qualify and/or quantify the contaminants during the production process. Some tests, for example tests on sterility or abnormal toxicity, are described in the European Pharmacopoeia, while other tests, e.g. tests to quantify DNA, viruses, and contaminating proteins are being discussed. This indicates that more research will be necessary, because little is known about the risk-factors, as well as the fact that the described tests should be improved.

The main risk factors of monoclonal antibodies and recombinant DNA technology products are:

1. DNA-content. The Dutch Health Council and the World Health Organisation allow a maximum concentration of 100 picogram DNA per milligram antibody, whereas the Food and Drug Administration permits a maximum concentration of 10 picogram DNA per milligram protein. Up till now, no satisfying test is available.
2. Viruses. Murine and retroviruses are not allowed in the end product. Most viruses can be detected by MAP or RAP tests, lactic dehydrogenase-virus will be detected by LDH enhancement, and the thymic virus will be determined by studying thymus abnormalities in neonatal mice. Retroviruses can be detected by several methods.
3. Purity of the product. Spontaneous mutations, degradation of the product, formation of dimers, polymers and protein aggregates, just as protein contaminants, can be detected by electrophoretic and/or chromatographic methods. The proteins are separated on basis of their molecular weight or isoelectric point.

The most appropriate way to detect monoclonal antibodies is by affinity chromatography. However, these columns fall under trade secret, and are only available for Registration Authorities.

Pharmacists, who are responsible for the quality of products intended for use in man, should consider the risk factors as mentioned above as well as the identity, concentration and formulation of the final product. Besides, the whole production procedure will be under the supervision of a pharmacist, assuming the guidelines of "Good Manufacturing Practice".

The identity of monoclonal antibodies is described in terms of its specificity. If an affinity column with the right antigen is available, this would be the simplest method to determine the identity of the product. However, these columns fall under the trade secret and are not widely available. Biological and non-biological assays are used to describe Mab's as well as rDNA technology products. However, these assays do have many disadvantages, especially that they are not unambiguous.

The concentration or quantification is usually expressed in (biological) activity per milligram protein, the so-called specific activity. However, it is very important to know that different assays could produce different results. It should be recommended to introduce international standardized tests, to avoid confusion. Likewise, one should introduce international and/or lab-standards, to exclude differences due to experimental circumstances. It is important to be conscious of the fact that the specific activity gives no information about the purity of the product.

If monoclonal antibodies are conjugated with a radio-active marker, a cytotoxic or another drug, special attention should be paid to the stability of the binding, undesired products derived from the binding-reactions, or the changed properties of both the Mab and the coupled material.

The formulation of the final product.

Both Mab and rDNA-products are proteins. Because of their highly individual characteristics, it is impossible to formulate conditions, under which every product will be stable. Attention should be paid to avoid denaturation and proteolysis, and also long-term storage could introduce problems. Proteins can be stored at 4 °C, but -20 °C or -70 °C are recommended, especially over long periods. However, most proteins are susceptible to denaturation by freeze-thaw cycles. Proteolytic degradation may be eliminated by lyophilization (freeze-drying), but many proteins are irreversibly denatured by the process. Proteolysis upon storage may arise from proteases in the sources or microbial contaminants. So purity of the product is very important.

Up till now little attention is paid to the administration of Mab and rDNA products. Both products being proteins, intravenous administration is the most appropriate one. The demands of intravenous administration are described in the European Pharmacopoeia (pH, salt concentration). In addition, one should be prepared for possible adverse reactions to the administered foreign proteins. The Dutch Health Council advises to administer Mab under the direct supervision of the attendant medical. It should be recommended to act with rDNA derived products likewise.

In conclusion, both Authorities (FDA, WHO, Dutch Health Council, EOQC), and manufactures (EFPIA, CLB) formulated guidelines and "points to consider" for the production and quality control of monoclonal antibodies and/or products derived by recombinant DNA technology. Up till now, specifically the hospital pharmacist is concerned with the quality control of these products. Because of the amount of the described tests, the quality control are likely to be done by or under the supervision of specialized laboratories. Partly to save expenses, partly because of the available material, apparatus, and knowledge.

Research should be focused on the (international) standardisation of the production procedures, the control of these procedures: demands, performance, and interpretation of the quality-tests. At the same time, research should be done to correlate

bioactivity with the (bio)chemical characteristics to establish the identity and the amount of the product.

**ABBREVIATIONS**

| | |
|---|---|
| AAI | N-acetoxy-N-acetylamino-7-iodofluorene |
| ABx | antibody exchanger |
| ATP | adenosine triphosphate |
| BCDF | B cell differentiation factor |
| BCG | Bacille Calmette Guerin |
| BCGF | B cell growth factor |
| BRM | Biological Response Modifier |
| CD | circular dichroism |
| Con A | concanavalin A |
| CSF | colony stimulating factor |
| DAPI | 4,6-diamidino-2-phenylindol 2 HCl |
| DEAE | diethylaminoethanol |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| E. coli | Escherichia coli |
| EFPIA | European Federation of Pharmaceutical Industries' Associations |
| ELISA | enzyme-linked immunosorbent assay |
| EOQC | European Organization for Quality, Pharma Section |
| FAB-MS | fast atomic bombardment - mass spectrometry |
| FACS | fluorescence activated cell sorter |
| FCS | fetal calf serum |
| FDA | Food and Drug Administration |
| HGPRT | hypoxanthine-guanine phoshoribosyl transferase |
| H(I)AT | hypoxanthine (insulin) aminopterin thymidine |
| HPLC | high performance liquid chromatography |
| HP-SEC | high performance - size exclusion chromatograhy |
| IFN | interferon |
| Ig | immunoglobulin |
| Il | interleukin |
| IRMA | immuno radiometric assay |
| kD | kilodalton |
| LAK | lymphokine-activated killer |
| LDH | lactic dehydrogenase |
| Mab | monoclonal antibody |
| MAP | mouse antibody producing |
| ug | microgram |
| mRNA | messenger RNA |
| Mw | molecular weight |
| ng | nanogram |
| NK | natural killer |
| PCA | passive cutaneous anaphylaxis |
| PEG | polyethylene glycol |
| pI | isoelectric point |
| r | recombinant |
| RAP | rat antibody producing |
| RIA | radio immuno assay |
| RNA | ribonucleic acid |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| TNF | tumour necrosis factor |
| UV | ultraviolet |
| WHO | World Health Organization |

## REFERENCES


1. Goding JW, " Monoclonal Antibodies: Principles and Practice", 2nd ed., Academic Press, London (1986).
2 Roitt IM, Brostoff J, Male DK, "Immunology", Gower Medicinal Publishing, London (1985).
3. Edmond SK, Grady LT, Outschoorn AS, Rhodes CT, Drug Dev Ind Pharm, 12, 107 (1986).
4. Ostlund C, Trends Biotechnol, 3, 288 (1986).
5. Kohler G, Milstein C, Nature, 256, 495 (1975).
6. Zola H, Brooks D, in: " Monoclonal hybridomaantibodies: techniques and applications. Hurrel JGR, ed., CRC Press, Florida (1982).
7. Tami JA, Parr MD, Brown SA, Thompson JS, Am J Hosp Pharm, 43, 2816 (1986).
8. Galfre G, Milstein C, Meth Enymol 73, 3 (1981).
9. Bartal AH, Feit C, Hirshaut Y, Dev Biol Stand, 57, 27 (1984).
10. Mason DW, Penhale WJ, Sedgwick JD, in: " Lymphocytes: a practical approach", Klaus GGB, ed., IRL Press, Oxford (1987).
11. Hopkinson J, Bio/technology, 3, 225 (1985).
12. Posillico EG, Bio/technology,,4, 114 (1986).
13. Lyderson BK, Pugh GG, Pans MS, Sharma BP, Noll LA, Bio/technology, 3, 63 (1985).
14. Bijsterveld MP, "In vitro production of monoclonal antibodies", Doctoral essay Pharmacy, Utrecht (1987).
15. Peters,Baumgarten H,Schule M, "MonoclonaleAntikorper: Herstellung und Charakterisierung, 2nd ed., Springer-Verlag, Berlin (1985).
16. Cohn EJ, Strong LE, Hughes WL et al., J Am Chem Soc, 71, 541 (1948).
17. Oncley JL, Melin M, Richert DA, Cameron JW, Gross PM, J Am Chem Soc, 71, 541 (1949).
18. Manil L, Motte P, Pernas P, Troalem F, Bohuon C, Bellet D, J Immunol Methods, 90, 25 (1986).
19. Neoh SH, Gordon C, Potter A, Zola H, J Immunol Methods, 91, 23 (1986).
20. Russo C, Callegro L, Lana E, Ferrone S, J Immunol Methods, 65, 269 (1983).
21. Swingle M, Tiselius A, Biochem J, 48, 171 (1951).
22. Juarez-Salinas H, Bigbee WL, Lamotte GB, Ott GS, Int Biotechnol Lab, April, 20 (1986).
23. Pavlu B, Johansson U, Nyhlen C, Wichman A, J Chromatogr, 359, 449 (1986).
24. Ross AM, Herlyn D, Koprowski H, J Immunol Methods, 20, 241 (1978).
25. Goding JW, J Immunol Methods,20, 241 (1978).
26. Fischer K, "An introduction to gel chromatography", North-Holland, Amsterdam (1969).
27. Pfannkoch E, Lu KC, Regnier FE, Barth HG, J Chrom Sci, 18, 430 (1980).
28. Weissenbruch F v., "Purification of murine monoclonal antibodies. Doctoral essay Pharmacy, Utrecht (1988).
29. Dick HM, Br Med J, 291, 762 (1985).
30. Conner CS, Drug Intell Clin Pharm, 18, 65 (1984).
31. Beverly PCI. Hosp Update, February, 223 (1983).
32. Central Laboratory of the Netherlands Red Cross Blood Transfusion Service. " Testing of mammalian cell products: monoclonal antibodies and rDNA products", Amsterdam, The Netherlands.
33. Pet, Centocor Europe BV, Leiden. Personal communication.
34. v. Hattem, Merck Sharp & Dohme BV, Haarlem. Personal communication.
35. Bult, Utrecht University, Faculty of Pharmacy. Personal communication.
36. Yalow RS, Berson SA, Nature, 184, 1648 (1959).
37. Oellerich M. J Clin Chem Clin Biochem, 18, 197 (1980).
38. Brown JP, Wright PW, Hart CE, Woodburg RG, Hellstrom KE, Hellstrom I, J Biol Chem, 255, 4980 (1980).
39. Frackelton AR, Rotman B, J Biol Chem, 255, 5286 (1980).
40. Secher DS, Burke DC, Nature, 285, 446 (1980)
41. Bottcher I, Hammerling G, Kapp J-F, Nature, 275, 761 (1978).
42. Eshar Z, Ofarim M, Waks T, J Immunol, 124, 775 (1980).

43. Bussard AE, Devel Biol Stand, 57, 13 (1984).
44. Monoclonale Antistoffen: toepassingen bij de mens. Eindadvies. Uitgebracht door een commissie van de Gezondheidsraad aan de Minister en Staatssecretaris van Welzijn, Volksgezondheid en Cultuur. The Hague, The Netherlands (1988).
45. World Health Organisation, Acceptibility of cell substrates for production of biologicals. Technical Report Series 747 (1987).
46. Rie MA de, Zeylemaker WP, Borne AEGK von dem, Out TA, J Immunol Methods 102, 187 (1987).
47. Weber K, Osborne M, J Biol Chem, 244, 4406 (1969).
48. Laemmli UK, Nature, 277, 680 (1970).
49. Wray W, Boulikas T, Wray VP, Hancock R, Anal Biochem, 118, 197 (1981).
50. Tomono T, Suzuki T, Tokunaga E, Anal Biochem, 123, 394 (1982).
51. Tomono T, Yoshida S, Tokunaga E, J Polym Sci Polym Lett Ed, 17, 335 (1979).
52. Committee for Proprietary Medicinal Products, Trends Biotechnol, 6, G5 (1988).
53. Thornton DH, Nicholas RAJ, Develop Biol Stand, 57, 9 (1984).
54. Der CJ, Clin Chem, 33, 641 (1987).
55. Carthew P, J Gen Virol, 67, 963 (1986).
56. v. Mourik, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service. Personal communication.
57. Shek WR, "Detection of murine viruses in biological materials by the mouse antibody production test".
58. Lubiniecki AS, May LH, Develop Biol Standard, 60, 141 (1985).
59. Monroe JH, Brandt PM, Appl Microbiol, 20, 259 (1970).
60. Pinteric L, Taylor J, Virology, 18, 359 (1982).
61. Lieber MM, Benveniste RE, Livingstone DM, Todaro GJ, Science, 182, 56 (1973).
62. Rowe WP, Pugh WE, Hartley JH, Virology, 42, 1136 (1970).
63. Manly KF, Givens JF, Taber RL, Zeigel RF, J Gen Virology, 39, 505 (1978).
64. European Pharmacopoeia 2nd ed. V.2.1.3.
65. Kaplan DR, Henkel TJ, Braciale V, Braciale TJ, J Immunol, 132, 9 (1984).
66. Chen TR, Exp Cell Res, 104, 255 (1977).
67. Pesce MA, Strande CS, Clin Chem, 19, 1265 (1973).
68. Lowry OH, Rosebrough NJ, Fair AI, Randall RJ, J Biol Chem, 193, 265 (1951).
69. Bradford MM, Anal Biochem, 72, 248 (1976).
70. European Pharmacopoeia 2nd ed. V.2.1.1.
71. European Pharmacopoeia 2nd ed. V.2.1.5.
72. European Pharmacopoeia 2nd ed. V.2.1.4.
73. Food and Drug Administration. Points to consider in the manufacture and testing of monoclonal antibody for human use. US Dept. of Health and Human Services, FDA, Bethesda (1987).
74. Kapuscinski J, Skocylas B, Anal Biochem, 83, 252 (1977).
75. Labarca C, Paigen K, Anal Biochem, 102, 344 (1980).
76. Wezel AL van, Marel P van der, Beveren CP van, Verma I, Salk PL, Salk J, Develop Biol Stand, 50, 59 (1982).
77. Hajduk SI, Science, 191, 858 (1976).
78. Brunk CF, Jones KC, James TW, Anal Biochem, 92, 497 (1979).
79. Rigbey PWJ, Dieckmann M, Rhodes C, Berg P, J Mol Biol, 113, 237 (1977).
80. Chou S, Merigan TC, N Eng J Med, 308, 921 (1983).
81. Barraud-Hadidane B, Martin RP, Montagnon B, Dirheimer G, Arch Toxicol Suppl, 11, 200 (1987).
82. National Control Laboratory-National Control Authority. Proposed Standard Registration Document for Monoclonal Antibodies (1988).
83. Prave P, Faust U, Sittig W, Sukatsch DA (ed.), "Basic Biotechnology, a student's guide. Weinheim, VCH Verlagsgesellschaft mbH, FRG (1987).
84. Stryer L. "Biochemistry", 2nd ed., WH Freeman and Company, San Francisco (1975).
85. Committee for Proprietary Medicinal Products. Trends Biotechnol, 5, G1 (1987).
86. The National Institute for Biological Standards and Control, London, Document, which is intended to provide a framework or discussion of points to consider in the manufacture and quality control of cytokines.

87. Food and Drug Administration. Points to consider in the production and testing of new drugs and biologicals produced by recombinant DNA technology. Office of Biologics Research and Review. National Center for Drugs and Biologics. FDA, Bethesda (1985).
88. National Control Laboratory-National Control Authority. Proposed Standard Registration Document recombinant DNA Products (1987).
89. European Federation of Pharmaceutical Industries' Associations (EFPIA). Production and quality control of polypeptide medicinal products derived from biotechnology (1986).
90. Teelman K, Hohbach C, Lehmann H. "Preclinical safety testing of polypeptide medicinal products derived from biotechnology". International Working Group. Publication EFPIA, Brussel (1986).
91. European Organization for Quality. Section for Quality Control in Pharmaceutical and Cosmetic Industries. Quality assurance in the manufacture of products from biotechnology. Conference organized by the EOQC Pharma Section in cooperation with David Begg Associates, York, UK. Frankfurt, FRG, December 1-2 (1988).
92. Janssen. Gist-Brocades, Meppel. Personal communication.
93. Pestka S, Pharm. Technol, Feb., 32 (1989).
94. Irwin MM, Oncol Nurs Forum, 14, 32 (1987).
95. Meager A, Leung H, Wooley J, J Immunol Methods, 116, 1 (1989).
96. Flick DA, Gifford GE, in "Biological Response Modifiers", Torrence PF ed., Academic Press, Florida (1985).
97. Bass R, Scheibner E, Arch Toxicol Suppl, 11, 182 (1987).
98. Food and Drug Administration. Points to consider in the characterization o cell lines used to produce biologicals. Office of Biologics Research and Review, FDA, Bethesda (1987).
99. Lubinieki AS, May LH, Develop Biol Stand, 60, 141 (1985).
100. Oldham RK. in "Biological Response Modifiers: new approaches to disease intervention, Torrence PF, ed., Academic Press,Florida (1985).
101. Mitchell MS, "Biomodulation: a classification and overview. Immunity to cancer", Academic Press, New York (1985).
102. Old LJ, Scient Am, May, 41 (1988).
103. Gresser IA. Chekhov M.D, N Engl J Med, 317, 457 (1987).
104. Carswell EA, Old LJ, Kassel RI, Green S, Fiore N, Williamson B, Proc Natl Acad Sci, 72, 3666 (1975).
105. Mannel DN, Moore RN, Mergenhagen SE, Infect Immun, 30, 523 (1980).
106. Mathews N, Immunology, 44, 135 (1981).
107. Aggarwal BB, Kohr WJ, Hass PE et al., J Biol Chem, 260, 2345 (1985).
108. Beutler B, Greenwald D, Hulmes JD et al., Nature, 316, 552 (1985).
109. Deventer SJH van, Debets JMM, Buller HR, Cate JW ten, Linden CJ van der, Buurman WA, Ned Tijdschr Geneeskd, 133, 17 (1989).
110. Beutler B, Cerami A, N Engl J Med, 316, 379 (1987).
111. Taverne J, Matthews N, Depledge P, Playfair JHL, Clin Exp Immunol, 57, 293 (1984).
112. Beutler B, Mahoney J, Le Trang N, Pekela P, Cerami A, J Exp Med, 161, 984 (1985).
113. Shirai T, Yamaguchi H, Ito H, Thodd CW, Wallace B, Nature, 313, 803 (1985).
114. Pennica D, Nedwin GE, Hayflick JS, et al., Nature, 312, 724 (1984).
115. Shalaby MR, Aggarwal BB, Rinderknecht E, Sverdersky LP, Finkle BS, Palladino MA, J Immunol, 135, 2069 (1985).
116. Gray PW, Aggarwal BB, Benton CV, et al., Nature, 312, 721 (1984).
117. Gray PW, in: "Lymphokines: Molecular cloning and analysis of lymphokines, vol 13. Webb DR, Goeddel DV, eds., Academic Press, Florida (1987).
118. Teppo A-M, Maury CPJ, Clin Chem, 33, 2024 (1987).
119. Sunahara N, Kurooka S, Kaibe et al., J Immunol Methods, 109, 203 (1988).
120. Isaacs A, Lindenmann J, Proc Roy Soc London B, 147, 258 (1958).
121. Stewart WE, Nature, 286, 110 (1980).
122. Dianzani F, Dolei A, Dev Biol Stand, 60, 3 (1985).
123. Langer JA, Pestka S, Immunol Today, 9, 393 (1988).
124. Pollard RB, Drugs, 23, 37 (1982).
125. Schellekens H, Pharm Weekbl, 116, 469 (1981).
126. Munk K, Kirchner H, in "Contributions to Oncology", Karger, Basel (1982).

127. Hubbard R, in: "Immunotoxicology". Gibson GG, Hubbard R, Parke DV, eds. (1983).
128. Paul WE, Immunol Today 9, 366 (1988).
129. The pleiotropic effects of B cell factors. Immunol Today, February, 9 (1988).
130. Oppenheim JJ, Kovacs EJ, Matsushima K, Durum SK, Immunol Today, 7, 45 (1986).
131. Hamblin AS, O'Garra A, in: "Lymphocytes: a practical approach", Klaus GGB, ed. IRL Press, Oxford (1987).
132. Gray PW, Glaister D, Chen E, Goeddel DV, Pennica D, J Immunol, 37, 3644 (1986).
133. Larrick JW, Immunol Today, 10, 61 (1989).
134. Gubler U, Chua AO, Stern AS, et al., J Immunol, 136, 2492 (1986).
135. March CJ, Mosley B, Larsen A, et al., Nature, 315, 641 (1985).
136. Yokota T, Arai N, Lee F, Rennick, Mosmann T, Arai K-I, Proc Natl Acad Sci USA, 82, 68 (1985).
137. Robb RJ, Kutny, Chowdhry V, Proc Natl Acad Sci USA, 80, 5990 (1983).
138. Robb RJ, Immunol Today, 5, 203 (1984).
139. Taniguchi T, Matsui H, Fujita T, et al., Nature, 302, 305 (1983).
140. Garland JM, Immunol Today, 7, 104 (1986).
141. Cosman D, Immunol Today, 9, 97 (1988).
142. Campbell HD, Fung H-C, Hapel AJ, Young IG, in: "Lymphokines", vol 13, Webb DR, Goeddel DV, eds., Academic Press, Florida (1987).
143. Yokota T, Otsuka T, Mosmann T, et al., Proc Natl Acad Sci, 84, 7388 (1985).
144. Yokota T, Coffman RI, Hagiwara H, et al., Proc Natl Acad Sci, 84, 7393 (1985).
145. Bazin R, Lemieux R, J Immunol, 139, 780 (1987).
146. Hirano T, Taga T, Yasukawa K, et al., Proc Natl Acad Sci, 84, 228 (1987).
147. Hirano T, Yasukawa K, Harada H, et al., Nature, 324, 73 (1986).
148. Hirano T, Taga T, Nakano N, Proc Natl Acad Sci, 82, 5490 (1985).
149. Wong GG, Clark SC, Immunol Today, 9, 137 (1988).
150. Namen AE, Lupton S, Hjerrild K, Nature, 333, 571 (1988).
151. Henney CS, Immunol Today, 10, 170 (1989).
152. Wing EJ, Shadduck RK, in: "Biological response modifiers: New approaches to disease intervention", Torrence PF, ed., Academic Press, Florida (1985).
153. Gaffney EV, Dell'Aquila ML, Lingenfelter SE, Huffnagle GB, Wiest DL, J Leuk Biol, 39, 409 (1986).