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SCIENTIFIC AND REGULATORY ASPECTS OF MACROMOLECULAR DRUGS AND DEVICES

M. D. Giddins[a], Roger Dabbah[b], L. T. Grady[b], C. T. Rhodes[a]

[a]Department of Pharmaceutics
University of Rhode Island
Kingston, RI 02881

[b]The United States Pharmacopeial Convention, Inc.
12601 Twinbrook Parkway
Rockville, MD 20852

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OBJECTIVES

1. To assess current and potential methods of large scale manufacturing of macromolecular products, including in process control and validation and assessment of final product.
2. To evaluate established and developing policies of regulatory agencies, research and compendial organizations concerning application of the technology and control of the final product; and to report on the legal situation regarding patents for biotechnology processes and products.
3. To discuss the expansion of the pharmaceutical industry into the field of biotechnology and the resulting implications of such a move on an established business.

1.0 Introduction

Advances in fields of genetics and subsequently genetic and protein engineering, in the past twenty-five years, have led to the development of new biotechnologies and a revolution in medical research that has the potential for producing an infinite number of pure, potent, and specific new protein drugs and devices.

1.1 Historical Background and the Development of New Technologies

From the discovery of the heredity function of chromosomes (circa 1930) genetics has progressed through the identification of

genes, the genetic material and the genetic code, and on to the understanding of mechanisms for the expression of proteins in cells.

By studying the genetics of bacteria and viruses, scientists in the 1960's identified plasmids and phages as the vectors that transferred genetic material from one bacterium to another. In 1973 came the discovery of restriction enzymes that provide a protective mechanism for the bacterium against foreign genetic material. These advances led to the application of plasmids, phages, and restriction enzymes as tools of genetic engineering. By the end of the 1970's researchers had learned to read the genetic code of certain proteins, synthesize their DNA (deoxyribonucleic acid) and insert it into bacteria so the protein could be produced.

In 1975 Kholer and Milstein, working with mammalian cell cultures, showed that by fusion of certain types of cells it was possible to produce genetic hybrids that would grow continuously in culture and produce unlimited quantities of homogenous, monospecific antibody.

As research to perfect these techniques continued, complementary, supplementary and even competitive technologies were developing in the fields of cell fusion, genetic and protein engineering. Application of these technologies heralded realization that here were methods of producing potentially endless medically useful proteins that could dramatically alter prevention, diagnosis and therapy of developmental and genetic disease.

All biotechnology projects, from research to commercialization, are multidisciplinary and incorporate such areas as molecular biology, biochemistry, microbiology, organic and physical chemistry and engineering. Preliminary assessment of the feasibility and viability of a project is necessary before discoveries on a laboratory scale can be applied to an industrial

scale. Not only must methods of production be addressed but also methods to characterize and standardize potential products.

Biotechnology has developed by utilizing new technology and applying existing technology from other established fields. The large scale manufacture of products is an expanding industrial concern and although some products are now well established the implications are still immeasurable and ideas will continue to develop along with expansion of the biotechnology industry.

2.0 Strategic Approach for the Production of Macromolecular Drugs and Devices

Relevant macromolecules could be known entities that exist naturally in certain environments, they may be completely novel entities, designed from first principles or they may be a combination of the two.

This section categorizes macromolecules as naturally occurring, modified natural products or manmade products, although as will be seen it is extremely difficult to define such categories.

When a product reaches the stage of development where application to large scale manufacture is necessary, technical, economic, purity and safety factors need to be considered. There are currently six methods by which a macromolecular drug or device could be obtained:

1. Extraction from natural source
2. Modification of naturally occurring protein
3. Mammalian cell culture in-vitro
4. Mammalian cell culture in-vivo
5. Production by microorganisms
6. Chemical synthesis

If we consider, for example, mammalian protein products, many are known to be naturally occurring but may also be produced by one or more alternative methods. The advantages and disadvantages

of each method must be studied before application to commercialization.

2.1 Natural Products

There are many naturally occurring macromolecules that have or could have medical application. They have been obtained by isolation from animal tissues or fluids, extraction from plants or from natural production by a microorganism. Availability of the raw material is often limited or variable. Raw material costs are generally low but there are extremely high processing costs involved. Isolation and purification can be complex and expensive and yield only very small amounts of the desired product.

Insulin and Human Growth Hormone are both naturally occurring proteins that have proven medical applications and provide good examples of the problems that can develop when utilizing a natural product.

There are naturally occurring products where new methods of production are not immediately required but there may be specific advantages in commercializing alternatives. There are also the products of potential medical use which are available in such small quantities naturally that alternative methods of production are necessary to ascertain whether the macromolecule could have useful application.

Some naturally occurring proteins can also be obtained by application to cell culture and fermentation techniques which are discussed later.

2.2 Modified Natural Products

There are methods of modifying proteins by combining information on crystal structure and protein chemistry with artificial gene synthesis. Also there are methods of adapting immunoglobulins, by protein engineering, for their application as reagents for affinity purification or as novel therapeutic agents. Because these methods incorporate some application of manmade entities they will be discussed later; here, we are considering

the possibility of altering one macromolecule to produce a second with differing characteristics.

Once a medically useful product has been completely characterized there is always potential for improvement by modification using design technology or structure activity relationships. For example, there would be advantages in improving efficacy and stability, increasing duration of action, potency and specificity or decreasing side effects. However, because the raw material itself is a naturally occurring macromolecule, any problems of supply, purification or yield improvement are still relevant. The cost of modifying the macromolecule is additional to the cost of obtaining its precursor and in the majority of cases the application of the project to a large scale is not viable economically, especially if alternative methods of production are available.

One area where there is current interest is in the modification of enzymes for industrial use. It would be extremely useful to be able to control enzyme properties such as thermostability, substrate specificity, co-factor requirements or pH optimum. Studies in structure activity relationships and the variation of enzymes of the same type, but from different sources, suggest modification would be possible. It is, however, unlikely that large scale application of conventional modification techniques would be economically or technically viable, especially if a number of specific amino acid changes distributed throughout the protein were required.

With the development of more extensive methods to modify proteins, specifically gene modification and synthesis techniques, it is unlikely that modification of a naturally occurring molecule will be as widely used as other methods for protein manufacture.

2.3 Manmade Products

With the expansion of recombinant DNA (rDNA), hybridoma, and protein engineering technologies the feasibility and viability of

production projects are needing increasingly detailed study. The interest and impetus for application of these technologies has developed because of the wide range of products possible, the potential for novel products, and the improvement of existing products.

Without the industry's success in bringing first generation biotechnology products to marketing and clinical evaluation there would be much less financial enthusiasm for expansion.

Because of the expertise and resources required many industrial companies are developing new strategies to overcome deficiency in these areas. These strategies include:

1. Collaboration with academic institutions
2. Internal expansion -- buying a small company -- expanding research facilities
3. Forming joint venture liaisons -- pharmaceutical companies with biotechnology companies

Expansion into biotechnology is still at its early stage and to increase the probability of success companies are focusing limited resources and prioritizing projects. It is also essential for industry to select the right opportunity and to direct research and development and marketing efforts accordingly.

2.3.1 Recombinant DNA (rDNA)

Recombinant DNA technology involves the systematic arrangement and manipulation of specific segments of nucleic acid for construction of composite molecules which when placed into an appropriate host environment will yield a desired product.

There are three general methods for obtaining a specific coding segment:

1. Reverse transcription of messenger Ribonucleic acid (mRNA) to complementary DNA (cDNA)
2. Isolation of genomic DNA or RNA
3. Chemical synthesis

Since many of the commercially valuable proteins are present in very small amounts in animal cells and tissues, much of the

commercial work so far has centered on the cDNA cloning of mRNA's present in cells. However, industrial production of Human Insulin was achieved with a chemically synthesized gene; it is likely that as the potential for production of novel proteins is realized this will become a more widely applied and improved method.

The technologies involved in producing a protein by rDNA are widely documented and will not be discussed in this review.

Most recombinant proteins have been produced in either E. coli or yeast fermentations. Large scale mammalian cell culture has also been applied to rDNA protein production. Animal cells are able to translate and process large or complex cloned proteins by the precise steps necessary to result in the correct disulfide linkages and glycosylation patterns.

Processes involved in commercialization of a rDNA produced pharmaceutical

1. Cloning of gene in laboratory
2. Subcloning of gene in organism or cell culture to achieve commercial levels of yield
3. Process development scale up
4. Large scale fermentation/cell culture development
5. Purification development
6. Animal testing
7. Clinical testing
8. Regulatory approval
9. Marketing

The complete procedure has been documented as possibly taking eight or more years to complete. However, Merck's rDNA Hepatitis Vaccine which was approved by FDA July 23, 1986, after a five month review, took only seven years from the first characterization of the virus gene until marketing. It is not unusual for new drugs to take this long for clinical trials and FDA review alone, the short development cycle for this recombinant product shows there is possibility for accelerated development as biotechnology products become more common.

The number of proteins that are attractive candidates for commercial production are endless, but most of the products being worked on today fall into four main categories: Hormones, Immunoregulatory Proteins, Blood Products, and Vaccines.

2.3.1.1 Hormones

Human Insulin

Human Insulin (Humulin), Genentech-Eli Lilly from E. coli was the first therapeutic recombinant product, approved by FDA September 1982. The A and B chains were produced separately from synthesized oligonucleotides, converted to S-sulfonated derivatives, purified, and joined by air oxidation to form active insulin. Details of the procedures used are widely documented (Johnson, I. S. (1983) and general references section 1).

Human Growth Hormone (hGH)

Methionyl Human Growth Hormone (Protropin)-Genentech was approved by FDA September 1985. Since approval it has been granted "orphan drug" status by the FDA. This allows Genentech a seven year monopoly on the drug's sales in the USA. Again technical methods used are widely documented (general references section 1), but one problem encountered during development was that the initiator methionine residue was not removed from the rest of the hormone. Possibly the precise sequence of the hormone chain and its folded structure prevented E. coli enzymes removing the residue.

Kabi Vitrum has received approval for its rDNA methionyl human growth hormone 'Somatonorm' in the U.K. and Belgium.

The rDNA product is established therapeutically, but some concern with antigenicity in a few patients does exist. Research into producing a rDNA human growth hormone without the additional methionine continues. Lilly has a product in clinical trials and Bio-Technology General (BTG) claims to have isolated an enzyme that selectively cleaves the methionine. BTG filed a notice of claimed investigational exemption for a new drug (IND) with FDA in

December 1985. A recombinant hGH produced by Serono from mammalian cells was predicted to start phase III clinical trials in Spring 1986.

Enkephalins and endorphins are being investigated as potential rDNA products along with various veterinary growth hormones now in trials.

2.3.1.2 Immunoregulatory Proteins

Interferons

The interferons are a family of cellular proteins (α, β and γ) produced by leukocytes, fibroblasts, and lymphocytes in response to viral invasion or to some RNA and foreign antigens. Early experiments with impure preparations led to inflated expectations which have only recently even begun to approach reality. Application of rDNA techniques has allowed identification of multiple interferon (IFN) genes. The necessity for so many different IFNs is not completely understood and further work is required to clarify their role and potential for therapeutic use.

Products of αIFN have recently surfaced and are now in use for certain specific conditions.

1. Intron A -- Schering licensed from Biogen, deglycosylated α IFN 2b was approved by FDA June 4, 1986 and by the Committee on the Safety of Medicines (CSM) UK for treatment of hairy cell leukemia only. Applications are also pending for its use in Kaposi's Sarcoma, multiple myeloma, malignant myeloma, venereal warts, and prevention of the common cold.

2. Roferon A -- Roche licensed from Genentech, deglycosylated αIFN 2a was approved by FDA June 4, 1986 for hairy cell leukemia and CSM (UK) for treatment of Kaposi's Sarcoma. Applications are also pending for cancer indications. Roferon A differs from Intron A by one amino acid. Both are manufactured using E. coli bearing a genetically engineered plasmid containing DNA which codes for human protein.

3. Wellferon -- Wellcome Foundation UK, αIFN-nL was approved by CSM (UK) for hairy cell leukemia only, it is a mixture of seventeen different αIFNs purified from a lymphoblastic cell line. Hence, the product is not manufactured by rDNA but its closer resemblance to natural mixed IFN may give it a broader spectrum of activity.

4. Immuneron -- Biogen γIFN in phase III clinical trials for renal cell carcinoma and phase II trials for ovarian carcinoma, chronic myelogenous leukemia, and malignant melanoma. The product has received a patent in West Germany and approval is expected for rheumatoid arthritis by end of 1986.

5. β IFN -- Phase II clinical trials reported in December 1985. Some success in reduction of disease symptoms of multiple sclerosis has been reported. Cetus Corp. has a recombinant βIFN analogue under test, "Betaseron."

Lymphokines

Lyphokines are only recently beginning to be characterized but are proving crucial to understanding immune reactions.

Interleukin-2 (Lymphocyte growth factor) is presently undergoing clinical trial to evaluate its potential to stimulate T-cells to become natural killer cells for destruction of tumors.

Cetus Corp. has a product in phase II trials for melanoma, colorectal, lung and kidney cancers. The product "Proleukin" is an analogue of interleukin-2 to attempt to improve action and gain proprietary position for the company.

Interleukin-2, Inc. (USA) have recently announced results of phase I trials of an interleukin-2 lymphokine natural "cocktail" product.

Other lymphokines, interleukins 1 and 3, and a number of regulatory "growth factors" for somatic cells are also candidates for rDNA production. Human Epidermal Growth Factor has been produced in yeast cells and Tumor Necrosis Factor (TNF) is under clinical trial. Cetus Corp. expects TNF approval by FDA around 1990.

2.3.1.3 Blood Products

Tissue Plasminogen Activator (t-PA)

Tissue Plasminogen Activator is probably the product produced by rDNA, with the most clinical potential at this time. An enzyme very effective in dissolving blood clots which may offer a new line of treatment for myocardial infarction, the leading medical cause of death in the USA. Other potential indications include pulmonary embolism, stroke, and various thrombotic disorders.

Genentech, probably the lead company in commercialization, cloned and expressed the gene in 1983 and entered clinical trials in February 1984. Application for FDA approval was submitted in July 1986. If FDA approves marketing by early 1987, t-PA could be commercially available less than two years after publication of the first major clinical trials.

t-PA has been produced in mammalian cell culture using a gene isolated from the Bowes Melanoma cell line. The Genentech product is from the Chinese Hampster Ovary cell line and therefore glycosylation patterns differ. Production of t-PA from a mouse cell line is also under development. The clinical effect of the different glycosylation patterns has not yet been established.

Research is already underway to attempt modification of t-PA to improve its action. Work has been reported of a t-PA encoded by a new DNA sequence with amino acid modification, cloned and expressed in *E. coli* and activated by proteolytic cleavage. The modification is reported to prevent complexation between amino acids 277 and 194 and results in a product with longer duration of action and slower blood clearance than known t-PA (half life: three to four minutes).

Kidney Plasminogen Activator (k-PA)

Another anticlotting compound has been developed by Collaborative Research under sponsorship from Sandoz. Approved for clinical trials k-PA is possibly more selective than t-PA in acting at coronary clotting sites. Experiments indicate t-PA and

k-PA may be synergistic -- they may be more effective and less toxic when administered together. A third clot dissolver, Aspac (anisoylated plasminogen streptokinase activator complex), is being developed by Beecham Group for single dose emergency treatment.

Blood Plasma Commodities

Includes serum albumin, gamma globulin and antihemophilic factors. rDNA albumin, expressed in 1981, is in clinical trial and Factor VIII expressed in 1984 is in animal studies.

Other rDNA protein products that are perhaps further back in development include: erythropoietin in trials for anemia; protein C, an anticoagulant, and its cofactor, protein S; and atrial naturetic peptide (ANP) for hypertension.

2.3.1.4 Vaccines

Vaccines in use today are generally produced using killed or attenuated organisms that produce antibodies specific to antigen molecules on the surface of the vaccine organism (active immunity). Vaccines constitute an historic advance in protection against disease but are not without problems:

1. Pathogens have survived inactivation processes and can produce the disease when used in a vaccine preparation

2. Conventional vaccines do not immunize against all strains of the pathogen. Genetic variability of many viruses requires multivalent antibodies for protection

3. Many vaccines need refrigeration and are therefore not stable in Third World countries where they are most needed

4. Contamination problems can occur with large scale production of pure virulent organisms

5. Many pathogens cannot be grown in culture

Since surface proteins of viral particles are the major antigens that produce immunity it should be possible to use proteins, rather than the virus particles, as vaccines (subunit vaccines). If the DNA sequence of relevant antigens on the

pathogen can be determined, utilization of rDNA technology could produce active vaccines with no risk of pathogenicity.

Advantages of rDNA Vaccines

1. Do not contain genetic material of the pathogen therefore cannot result in infection

2. Are more stable and of considerable greater purity

3. Do not require large scale propagation of highly infective organisms

Use of rDNA techniques could also help to establish methods of designing combination vaccines that could combat problems of genetic variability and multiple strains -- polytropic vaccines.

Recombinant vaccines are being developed mainly using E. coli, Candida albicans, Saccharomyces cerevisiae, and Vaccinia virus systems. Much research is being targeted at engineering antigen-coding DNA into living microorganisms that could actually grow inside the host being immunized. Vaccinia virus, which is nonpathogenic in humans, has been successfully adapted to act as a carrier for several antigens.

Hepatitis Vaccine

Recombivax HB -- Chiron Corp./Merck was approved for vaccination against Hepatitis B virus July, 1986.

A Hepatitis B vaccine has been available since 1981 but is derived from plasma of infected patients. The process takes several months, creates risks for those involved in production and offers a limited supply. The Merck rDNA vaccine, expressed in yeast cells, can be produced in a few weeks and the technology allows unlimited supply. The new vaccine will be available in January 1987 and will cost approximately the same as the existing one.

Research into a vaccine for Hepatitis A, a milder disease, is also underway. Attenuated live vaccines are in pre-clinical trials and killed whole virus vaccines are in early clinical trials. Research is also current to construct viable, full length

cDNA clones which could be modified by site-specific mutagenesis and to express Hepatitis A virus capsid proteins in prokaryotic or eukaryotic systems.

AIDS Vaccine

Genentech and Harvard Medical School researchers have reported production of recombinant antigens from an envelope glycoprotein of the AIDS retrovirus. The protein has been obtained from genetically engineered Chinese Hampster Ovary cells, but it will be some time before any relevant toxicity data is available.

Developments for an AIDS diagnostic test and an AIDS vaccine are still in very early stages. Any practical vaccine will also have to encounter the many variant forms the AIDS virus is known to assume.

Other Current Viral Vaccine Projects

1. Influenza vaccine
2. Polio vaccine
3. Herpes Simplex I and II and cytomegalovirus vaccine
4. Foot and Mouth vaccine
5. Pasteurella vaccine
6. Rabies vaccine

Bacterial Disease Vaccines

Because bacteria have complex cell surfaces, derived from multiple biosynthetic pathways, they are not readily amenable to vaccine production by rDNA. The biosynthetic pathways are controlled by large multiple genes. If the identity of unique proteins on the surface of bacteria could be established, subunit vaccines against bacterial disease could be possible.

Parasitic Disease Vaccines

Many parasites are protected from the immune response of their host by constant modulation of surface antigens.

Recent work on Plasmodium vivax has identified a peptide fragment that is constant amongst strains and binds to the surface proteins antibody providing protective immunity.

Chiron Corp., in collaboration with New York University, have produced a rDNA antisporozoite vaccine for P. vivax expressed in yeast cells and toxicity tests are in progress.

Human trials of an antisporozoite vaccine, developed by the Department of Health and Smith Kline and French Laboratories, started in March 1986. This vaccine was produced in E. coli using recombinant techniques.

Many of the areas under current research using rDNA techniques have been mentioned, however, the versatility of the technology suggests other applications are possible and no doubt many are being investigated. It is very likely that with the improvement of protein engineering techniques many of the smaller proteins and peptides may be manufactured more economically by chemical protein synthesis.

One application of rDNA protein production that has not been expanded to any great extent as yet is the potential for designing and manufacturing novel proteins by building synthetic genes.

2.3.2 Hybridoma

Established methods for production of hybridoma cell lines producing unique antibodies require the successful completion of a number of individual steps. Lymphocytes from immunized mice are chemically fused with myeloma cells from a suitable myeloma cell line maintained in culture. The resultant hybrids are propagated, screened, cloned, and scaled up to produce useful quantities of antibody product which need purification and characterization.

There are basically two methods of producing monoclonal antibodies from hybridomas: in-vivo culture as ascites tumors in mice and cell culture in-vitro. These methods will be discussed further in section 3.

The detailed technologies involved in producing hybridomas have been described by many authors along with applications for use of monoclonal antibodies as in-vitro diagnostics, for in-vivo imaging and in potential therapy in humans and animals (Nowinski

et al. (1985), Scott, M. G. (1985) and general references section 1).

The potential for applications of monoclonal antibodies (MAbs) is staggering but the use of the new technology for manufacturing medical products is evolving slowly. The status of the MAb product market at this time is mainly concentrated in in-vitro diagnostic products, possibly because of this field's low barriers of entry. There are currently over 60 MAb diagnostic products in widespread use (Scrip July 1986), but application of MAb technology to therapeutic use is still at a very early stage.

From studying the progress of the hybridoma industry it can be seen that developments were strategic from the start. Most of the first in-vitro MAb diagnostic products approved were in areas where diagnostic tests already existed. Once the greater speed, specificity, and reproducibility of the MAb products had been proven and accepted there was confidence to search for new markets and the industry developed further products for novel diagnostic applications.

Hybridoma technology for industrial application, such as immunopurification, is also now an established area and MAbs for use in in-vivo imaging are recognized, but their potential for direct or indirect therapeutic use is not yet being realized as quickly as originally hoped. There are persisting problems that must be resolved before the full clinical effect of MAbs can be measured.

Problems in Producing MAbs for In-Vivo Therapeutic Use

1. Obtaining MAbs of high specificity and affinity often to very weak immunogens or antigens that are only available in very small amounts

2. Obtaining MAb of predetermined class or subclass

3. Generating human MAbs to overcome antigenicity problems

4. Overcoming inherent characteristics of immunoglobulin molecules such as multivalency and ability to bind to Fc receptors

on cells which theoretically may diminish their effectiveness or even cause damage

5. Validating macrotechnology, quality assurance and control to improve purity and ensure sterility

The problem of generating antibodies of high affinity of a specific class places doubts as to their direct use in treating malignancies or their indirect use by conjugation in diagnosis or therapy. Only a small percentage of low affinity antibodies would bind and remain attached to the tumor. Therefore, large amounts of product would have to be injected. If antibodies that are either radioactively labeled or conjugated to a cytotoxic moiety do not have high affinity or specificity then many will stay in circulation, increasing background radiation or distributing the cytotoxic moiety to other areas of the body. The class or subclass of MAb used can determine effectiveness of binding, and studies in-vitro have shown that some classes are more effective in mediating macrophage dependent cytotoxicity.

Currently mouse MAbs are being used to a limited extent in human clinical trials. Some results are encouraging but applications are limited because the fact remains that mouse proteins are immunogenic in humans and patients can develop cross reacting antibodies.

There are research programs underway to try and alleviate these problems including technical modifications to increase yield of hybridomas. Improvements in screening techniques have allowed MAbs of desired specificity, affinity, and activity to be identified, but there are two recent advances in hybridoma technology that may improve the status of MAbs as therapeutic agents.

The first approach is the method of chemically fragmenting existing MAbs to produce F (ab') fragments which will bind antigen but will not bind to Fc receptors. These fragments clear rapidly from the circulation and are degraded. They will probably prove

useful in targeting agents to tumors but are less likely to be used directly in therapy.

The second approach incorporates the use of rDNA technology. It is possible to change the structure of the genes encoding a useful MAb to make it more effective and then introduce the modified gene into an animal cell or microorganism to produce large quantities of antibody. Unfortunately much further work needs to be done to apply this technique; little is known about which amino acid sequences are relevant to antigen binding and effector functions. It may be possible to identify rare hybridoma clones that undergo rearrangement, deletions or somatic mutations so that the structure of their genes and antibodies may be studied and related to function.

Application of rDNA technology may provide methods of making mouse MAbs less immunogenic in humans. It is possible to manufacture human hybridomas but the efficiency of transformation is low and the stability of MAb production variable. Human myeloma cell lines, although available, are difficult to maintain in culture and there are problems in obtaining human B cells at the right stage of differentiation.

Researchers using recombinant techniques have cloned variable region genes from mouse antibodies and joined them to genes of the human constant region. These chimeric genes are placed in nonimmunoglobulin producing mouse myeolma cells that will secrete hybrids with, in theory, the specificity from the mouse region with the functions of the human region. Other forms of chimeric molecules have been constructed and there is potential for producing many bifunctional chimeric monoclonal antibodies. Recombinant DNA techniques may also solve the problem of obtaining MAbs from malignant cells.

There are good indications that these strategies will improve the prospects of MAbs for in-vivo use, and it is possible that

therapeutic MAb products will eventually attain the market potential reached by the diagnostic products.

Two therapeutic products using new antibody technology have recently been approved by the FDA for in-vivo use, perhaps this is only the start of a revolution in therapy.

Orthoclone OKT 3 - Ortho Pharmaceuticals

Approved by the FDA June, 1986, two years after filing application, Orthoclone OKT 3 is a monoclonal antibody product indicated in kidney transplant rejection.

Digibind -- Burroughs Wellcome.

Digibind is digoxin immune Fab, an antibody fragment product specific for digoxin approved May, 1986 and indicated as an antidote for intoxications of digoxin.

2.3.3 Chemical Synthesis

At this time, a major factor in deciding which technology is suitable for large scale protein production is the length of the amino acid chain.

Modern methods of chemical synthesis (classic condensation methods or stepwise solid state synthesis) have resulted in increased yields at each synthetic step with the reduction in formation of side products, but there are many considerations that have to be taken into account if organic synthesis is to be commercially viable:

1. Cost of pure amino acid subunits plus chemicals used as activating, protecting, coupling, liberating and supporting agents

2. Cost of separating desired product from the supporting resin, by-products, and excess reagents

3. Cost of purification (generally less than for biologically produced proteins)

4. Cost of labor, plant, and equipment (synthesis involves many individual steps. The total time for completion is dependent on length of amino acid chain)

Some simpler proteins have been chemically synthesized and a few are being marketed, for example, calcitonin, a 32 amino acid peptide for the treatment of osteoporosis and Paget's disease. At the moment, higher molecular weight proteins and glycosylated proteins cannot be produced by chemical synthesis. The small peptides are those that will provide impetus for expansion of the method on a commercial scale once amino acids become cheaper and synthesis is economically viable.

Subunit vaccines are specific examples of good candidates for chemical synthesis. A subunit vaccine for foot and mouth disease is being developed in this way, linking the synthesized antigen to a protein carrier to increase immunogenicity. Other good candidates are peptide fragments that produce activity comparable to, or greater than, the parent protein.

The greatest challenge is that of designing and creating novel functional proteins from first principles. The only way that potential for this idea will be realized is by improving knowledge of protein structure related to function.

Although there are many established methods for sequencing proteins, it is still not possible to predict tertiary structure of a protein from its amino acid sequence. Folding patterns are critical to a protein's function and no two proteins fold in exactly the same way.

Modification of proteins can be used to correlate experimentally observed differences in structure with different functional properties. HPLC methods and two dimensional proton NMR techniques are also being used to provide information on structure. One great advancement is the use of computer graphics for model building and interactive computer graphics for designing.

It is necessary to develop a workable theory that would allow us to calculate the effects of small changes in amino acid sequence, thus eliminating the necessity to perform experiments at

each modification stage. There are no proven procedures as yet for de novo protein design, and we are a long way from building proteins that have designed biological and catalytic activity.

Gene Synthesis

The chemistry of DNA synthesis has advanced to a point where oligonucleotides are considered as standard laboratory reagents. They have already been utilized in the rDNA industry and will become more common in this field as novel proteins are developed.

In most cases primary sequence analyses of large proteins can be obtained more efficiently by DNA sequencing if the DNA coding for the protein can be cloned and identified.

Genetic engineering of oligonucleotide chains can also be used to study the structure activity relationships of proteins.

With the advances in protein engineering, genetic engineering, rDNA, and hybridoma technologies it is likely that novel proteins will eventually be produced for therapeutic use. Any substance that is not a natural constituent of the human body may be antigenic and may also cause unknown and possibly adverse biological effects. The use of such products in humans depends on careful assessment of benefits compared to risks identifiable during pre-clinical and clinical evaluation.

3.0 Manufacturing Techniques

There are two critical areas in the biotechnology industry where concern has developed as to technological shortcomings: scale up technology and downstream processing. Manufacturing techniques are relatively simple on a laboratory scale and already specialist companies which apply large scale manufacture are emerging. The genetic engineering that is applied to cells and organisms changes their characteristics and any problems on a small laboratory scale are enhanced dramatically when commercialized. It is anticipated that new problems could emerge.

The extent of scale up required must also be considered. For example, some products may only be therapeutically required in very small amounts while others may have larger market potential.

Problems in scale up may require consideration for involvement of specialized contract manufacturers, but the cost of developing relevant technologies has been high and most contract organizations are seeking joint ventures or some other profit sharing arrangement. The in-house approach requires that a manufacturing facility be built -- about $10 to $40 million in capital -- and that process development, a laboratory development group of say ten people at a total cost of $125,000 per person/year, clinical and sales groups be established (Genetic Engineering News, July/Aug. 1986).

3.1 Fermentation

Many factors that directly influence production of rDNA proteins have to be considered at the development stage, these include:

1. Gene dosage
2. Structure of promoter
3. Ribosome binding site
4. Stability of plasmid
5. Stability of mRNA
6. Stability of product
7. Effect of product on host cell metabolism
8. Choice of host coordinated to fit expression vector

Proteins have been expressed in various hosts including *E. coli*, the genetics and physiology of which are well characterized, yeasts which have a documented fermentation history and can glycosylate proteins, and Bacillus strains which like yeast can secrete proteins.

3.1.1 Master Cell Bank

Before large scale production a Master Cell Bank (MCB) has to be established. The MCB is a designated seed lot from which all

subsequent seed lots are made. A seed lot consists of aliquots of a single culture, stored to ensure genetic stability. In most cases a single host cell containing the expression vector should be cloned to give rise to the MCB. The identity and purity of cells in each seed lot should be assured and each seed lot should be characterized for adventitious agents.

3.1.2 Fermentation Equipment and Containment

Antibiotic fermenters were designed to exclude contaminating organisms. It has been necessary to modify them for containment of recombinant cells. The design of a fermenter on a large scale involves consideration of biological and engineering requirements.

Generally the vessels are closed cylindrical tanks containing agitators, baffles, heat exchange coils, and automatic controls for temperature, air flow, pressure, pH and foaming. Vessels in routine use can contain up to 50,000 liters and are constructed usually of stainless steel but borosilicate glass and inert polymers have been used. Designs are finely engineered to guard against leaks and dead spaces. The design of the agitator is critical for it must effect mass and heat transfer, aid in aeration, as well as mixing without harming the cells. Compressed air, filtered to remove contaminants, is pumped into the fermenter to provide oxygen for growth; metabolic heat is removed by cooling jackets and operating pressure is generally about 5 psig.

The need for containment is implemented by incineration or filtration of the exhaust gases, and sampling and inoculating with a closed system. To ensure integrity of the agitator seal, double mechanical seals must be installed. Verification of complete inactivation is carried out prior to disposal of any spent fermentation broth through normal waste handling channels.

Before use the whole system must be validated to ensure correct function and containment, and then sterilized. At the end of the process, before processing, the fermenter contents are chemically or thermally sterilized.

Although stirred tank reactors are the standard of the fermentation industry, because of their versatility, they have high energy requirements, can cause shear damage to cells, and there are local fluctuations in oxygen and carbon dioxide concentrations.

Other available fermenter designs:

1. Airlift fermenter -- This forces air through a sparger. It has advantages of low shear, low energy requirements and simplicity of construction; however, mixing can be poor.

2. Packed bed reactors -- These have found limited use and are generally only applied with immobilized cells and enzymes.

3.1.3 Reaction course

Various fermentation protocols, representing different manufacturing strategies, are used to obtain and maintain a high rate of product synthesis for as long as possible:

1. Batch Process -- All nutrients are loaded into the fermenter at the start of the process. Process continues until all nutrients are expended

2. Fed-Batch Process -- Nutrients are added as organisms grow. Growth is limited and controlled

3. Continuous Process -- Nutrients are constantly added and waste is removed

Single batches have advantages of design simplicity and flexibility and fed batches can be easily controlled. Continuous growth processes are more economical and, in theory, produce more product due to few interruptions and an increased number of cell divisions. Choice of reaction course is dependent on economical, technical and biological circumstances.

3.1.4 Conditions, Media and Raw Materials

Type of medium depends primarily on the microorganism being grown and in many cases the particular strain used. Cost of various components of a nutrient medium can amount to 40--70% of the total cost. Generally the carbon source, in the form of

carbohydrate, is the most expensive ingredient and its conversion efficiency (amount of product formed relative to maximum theoretical yield) for a highly expressed rDNA product is only in the region of 10%. Changing the substrate to another carbon source may give higher yields, but may also require more agitation or aeration with corresponding higher energy costs.

Functions of Medium

1. Source of elements
2. Source of nutrients
3. Source of energy
4. Heat and waste removal
5. Metabolic control

Choice of medium is one of the major considerations in the process. Chemically defined media are expensive but result in more economical downstream processing, and it is easier to control the stability of the organism and duration of the fermentation. The most common medium for E. coli fermentation, L-broth (yeast extract, tryptone and sodium chloride) has two expensive components but is reported to give good bacteria growth. If the product is intracellular and disruption of cells is necessary then a less defined medium may be used. If the cells secrete product there is incentive to work with defined media. Maintenance of the correct medium is essential to prevent intracellular protease enzymes which can be induced by starvation of cells.

Oxygen, pH and temperature conditions are critical. In most fermentation operations, a limiting factor is that the oxygen transfer capability of the system is exceeded ultimately by the oxygen demand of the culture. This results in the limitation of growth in fed-batch or continuous culture. Recent experiments in which no nutritional limitations were imposed have been described. The demand for nutrients was limited by the control of growth temperature. Growth at lower temperatures can increase cell yield.

3.1.5 Product Yield

It is necessary to determine the conditions such as pH, oxygen level, temperature and nutrient concentration that are key factors in the production of high yields. For many bacterial fermentations a maximum growth rate does not necessarily result in maximum synthesis of product; it must be known if product formation is either growth or cell yield dependent.

A recombinant gene product is synthesized in response to the rDNA gene originally cloned into the appropriate vector. It is difficult to maintain plasmid stability over extended periods of time, and once plasmid free cells are produced they are generally dominant; however, adaptive changes can occur which increase the growth rate of the plasmid containing cells resulting in delayed loss of plasmid from the organism population. Selective pressure to fermentation medium or altering growth conditions can maintain the rDNA gene in the population; but it is preferable to use an inducible promoter system that enables delay of high level expression until the end of the growth cycle (in single batches), so that mutations will only constitute an insignificant fraction of cells.

In the development stage of rDNA protein production it is very important to define host/vector combinations to ensure correct control of expression which might be inducible, end product regulated or temperature regulated.

3.1.6 Monitoring the Process

Maximum yields are the result of maintaining a set of ideal conditions. Process control requires automatic maintenance of conditions, and automatic changes in operating parameters in response to external or internal events.

Fermenter temperature, air flow, pressure, pH, dissolved oxygen, feed rates, and composition of exhaust gases all need to be controlled, and monitoring of the operating environment and

integrity testing of filtration systems should be incorporated into procedures.

The automated system must be able to respond to unexpected events by raising alarms, halting the process or providing data.

For these reasons computer systems are utilized as monitoring, controlling, data storage and processing agents. Computer compatible sensors are used in the process, and monitoring can ensure correct procedure and maintenance while also being able to recognize fermentation or containment problems whilst they are still correctable. There is, however, some need for development of applicable biosensors for on-line techniques. The lack of suitable sensing devices, to measure accurately the concentrations of biomolecules, leads to a combination of on-line and off-line control. Two methods for monitoring protein formation that are being developed are fast protein liquid chromatography and the use of characteristic biochemical reactions.

3.1.7 Recovery of Product

Some products produced by recombinant microorganisms are secreted into the medium. Under these circumstances product recovery is by separation and purification which will be discussed in section 4. The majority of products, however, are produced and maintained within the cells. Recovery of intracellular material therefore requires that either the cell is genetically engineered to secrete the product or that the cell is disintegrated by physical, chemical or enzymatic means. Currently the latter is the more common form for product recovery. One alternative that is being investigated is to chemically permeabilize the cell employing a nonionic detergent (Triton X 100) and a chaotropic salt (guanidine hydrochloride). This would decrease problems in purification where cell fragments, nucleic acids, and extraneous cell proteins have to be removed.

There are many methods for cell disruption, summarized as follows:

1. Mechanical methods with large scale applications:
   Liquid shear -- high pressure homogenizer
   -- ultrasonication
   Solid shear -- bead mill
   -- freeze press
2. Alternative methods with little large scale application at this time:
   Osmotic shock
   Enzymatic lysis -- although delicate and specific is limited by cost
   Chemical lysis -- nonselective, can cause contamination problems and damage proteins

The most widely used methods are the mechanical methods. Combination methods may prove useful for organisms resistant to disruption where mechanical methods alone become costly. The process method cannot be considered alone and the application of downstream processing must be taken into account because of possible changes in viscosity, density, and particle size of slurries.

Fermentation conditions can influence the resistance of cells to disruption. This is being investigated and may eventually be used favorably.

3.2 Cell Culture

Mammalian cell culture is the only commercial method, at the moment, for recombinant proteins needing specific conformations or other post translational modifications (for example, gamma carboxylation, glycosylation and activation) that cannot be achieved in microbial fermentation systems. If potential rDNA product candidates are not characterized they may also be manufactured by cell culture in order to produce sufficient amounts for investigational purposes.

Cell culture of hybridoma lines has become the more popular method for large scale production of MAbs. MAbs can be produced with reasonable efficiency in mice but the need for pure antibodies, free from murine contaminants, and the potential for human MAb production necessitates the use of cell culture.

Because of the large number of methods available no one technique will be specifically discussed in detail; many aspects discussed under fermentation manufacture are relevant to manufacture of recombinant proteins by cell culture.

3.2.1. Master Cell Bank

As with fermentation the manufacturer's production should be based on the use of a cell seed system. The cell seed lot is a quantity of cells stored frozen at -70°C or below. The identity and purity of each seed lot must be assured and each lot should be characterized for agents such as mycoplasma, bacteria, fungi, viruses, and virus-like particles.

3.2.2 Cell Culture Reactors and Reaction Course

Sophisticated bioreactors using novel systems as well as conventional fermenters are used. The choice of system can depend on whether cells are anchorage-dependent cells (ADC) or anchorage-independent, on economic considerations, and on product yield considerations.

Complex systems are necessary because mammalian cells grow at slow rates (average cell doubling time 18-48 hours compared to 20-30 minutes for bacteria), they are larger, more fragile, and more complex than bacteria; and their nutritional requirements more stringent and not well defined at present. Cell cultures are also very susceptible to contamination.

Hybridoma cell lines are anchorage independent as are those cells originating in blood and lymphatic tissue. The majority of cells are, however, anchorage dependent. Recombinant products are generally only expressed satisfactorily in ADC's.

Modified Conventional Fermenters. For anchorage independent cells only. Modifications necessary include an alternative

agitation system to reduce shear forces. Marine type impellers, vibromixers or rotating flexible sheets are used. Cell population densities are relatively low. This approach has been successfully used for hybridoma cell lines.

Air Lift Fermenters. Applied to submerged cell cultivation because of the low shear forces involved. Hybridoma cell lines of mouse, rat, and human origin have been grown in air lift fermenters. At Celltech, air lift fermenters of 10, 100, and 1,000 liters have been operated in cascade for production of MAbs. There appears no specific advantage in hybridoma culture over modified conventional fermenters.

Rotating Vessels. Rotating vessels of spin filter design have also been applied to hybridoma culture. Filters prevent cells from exiting in the effluent, but application appears limited as filters become blocked by cells and cell debris.

Various methods have been developed to immobilize cells for anchorage dependent growth. Area to volume ratio can be increased by various methods. Cells can be grown on spongy polymers, ceramic matrices, arrays of thin tubing or hollow fibers, on stacks of thin plates or on microscopically small beads called microcarriers. Many of these immobilization methods can be applied for use in slightly modified conventional type fermenters.

Microcarrier Beads. These are made of the natural glucose polymer, dextran, synthetic polymers or glass. Microcarrier beads can range in size from 50μm to several hundredμm in diameter. Beads are suspended, therefore agitation is still required. Bead density is of great importance, the surface to volume ratio must be high, but if density is increased too much a high collision rate results and cells are damaged; aggregation could also occur by growth bridges between beads.

Hollow Fiber Reactors. Hollow fibers, diameter 0.3 to 0.8 mm, are excellent mass transfer devices. They are packed in bundles within a cylindrical vessel and medium is delivered

through the lumens. Cells grow in extracapillary spaces. Fibers are made of semi-permeable materials. Some problems can occur due to pressure drop across the ends of the fibers.

In single batch cell cultures growth and cell life are limited due to depletion of nutrients and build up of wastes. One of the disadvantages of cell cultures is that they cannot be maintained in conventional continuous process. The nearest analogy to continuous processing is the application of perfusion. Perfusion systems can be applied to ADCs or anchorage independent cells, but the cells have to be trapped in some contained system to which fresh medium is added while spent medium and wastes are removed at the same rate.

Advantages of perfused systems: (can lead to lower capital costs with higher productivity and improved downstream processing)

1. Physiological state of cells can be maintained and manipulated to a greater extent

2. Frequently, the product is collected continuously in the spent medium

3. Cell densities attainable are greater than with conventional methods and can be regulated by biomass feedback systems

Disadvantages of perfused systems:

1. More complex than conventional systems

2. Need constant large supplies of medium

3. Have greater vulnerability to contamination and cell instability because of increased culture operation time

Perfusion cell cultures have run at 1,000 liter scales but most systems in use involve much smaller working volumes. It is too soon to predict the full potential of perfused culture systems.

Novel Systems Developed

1. Endotronics (Coon Rapids, MN) have developed a hollow fiber system with an expansion chamber to effect ultrafiltrative cycling of the culture medium

2. Bio-Response (Hayward, CA) have used a perfused hollow fiber system using more open porosity in mixed cellulose fibers

3. Static Maintenance Reactor -- licensed to Invitron - cells are grown in a perfusion chemostat system and then concentrated and immobilized with nontoxic matrix material. The cells, whether grown on microcarriers or in suspension, are mixed with the matrix

4. Opticell Division of KC Biologicals have developed the Opticore Ceramic Matrix to immobilize cells. Matrices for adherent cells have smooth surfaces while those for suspended cells have rough surfaces

5. Bio-Response have also used a packed bed of glass beads in a chemostat system

6. Verox (Hanover, NH) have developed a weighted sponge matrix formed into spherical beads for both ADCs and anchorage independent cells

7. Damon Biotech developed probably the most widely applied novel system and patented the process in 1978. The system involves incorporating cells into microcapsules. Microencapsulation can be applied to both recombinant protein and MAb production and has been routinely used for production of multigram amounts of MAbs. Cells are encased in a semi-permeable polymer membrane. The microencapsulation process is by mild chemical methods. Cells are immobilized in sodium alginate spheres which are coated with a bipolymer. Encapsulated cells are used in a conventional bioreactor at 37°C. The permeability of the membrane can be varied, for example, for either IgG antibodies (150,000 daltons) or IgM (900,000 daltons). Over 80 different cell lines have been grown in microcapsules by Damon Biotech.

3.2.3 Media, Raw Materials and Conditions

Cell culture media often contain over 50 individual components. They are usually basal salt solutions (carbohydrates,

amino acids and minerals) supplemented with peptones and animal serum to provide hormones, lipids and growth factors.

Serum is expensive, carries large risk of contamination, and supply is variable. If the products are for therapeutic use then the presence of serum proteins is undesirable. There is a trend towards more defined media, removing or reducing the serum content. (In theory serum can be used for microencapsulated cells without contaminating the product.)

A number of hybridoma lines can be supported with addition of insulin, transferrin, ethanolamine, and selenium; this has resulted in improved downstream processing. Work with recombinant cell lines shows differing medium requirements especially in serum free media. Differences occur between clones from the same line expressing different proteins or even mutations of the same molecule. The reason why this happens is presently unclear but makes a generic medium unlikely for the immediate future.

Variability of nutrient requirement of cells in different growth phases and culture systems must be taken into account when quantifying nutrients, estimates need to be related to cell mass or dry weight rather than population density.

Oxygen can be sparged into the vessel or a flow of air can be directed through semi-permeable membranes. Sparging is simple and efficient but can cause foaming and cell lysis. The alternative is to fit the vessel with semi- permeable tubes through which oxygen is passed.

pH is controlled generally by a carbon dioxide-bicarbonate buffer technique (as with fermenters). Oxygen level and pH are more difficult to control in higher density cultures where pH is better regulated using a meter to add base directly.

Foaming can result from sparging gases or vigorous agitation and is exacerbated by serum. This can cause major problems, for example, trapping microcarrier beads and damaging cells. Foaming should be controlled with antifoaming additives or foam trapping

devices (some cells cannot tolerate antifoams and antifoam substances may complicate downstream processing).

Contamination is a major hazard in cell culture especially when antibiotics cannot be used. Complexity of media, support systems, slow growth rates, and operational manipulation all increase risk of contamination. Mycoplasma pose a major threat as they are not easily detected and are highly contagious. Viral contamination is generally not a problem although certain infections such as parvovirus can have long latent periods before degenerative and lytic effects become apparent. Sources of viral contamination are animal serum and inadequately screened cells. Cell culture media need to be sterilized by filtration through depth or membrane filters and their ingredients must be strictly quality controlled. Equipment is steam sterilized before use and processing must utilize strict aseptic procedures. In a batch system limiting factors are often asepsis maintenance, buildup of metabolic by-products, and depletion of nutrients. Production of lactic acid can be decreased by substitution of glucose with galactose. Problems generally are mitigated by perfusion.

Cell growth rate, product formation, degree of activation, and viability depend on nutritional environment, environmental factors (oxygen, pH), and toxic contaminants.

3.2.4 Monitoring the Process

Generally the same procedures as those discussed in fermentation apply although there are more variables that need monitoring and control. Glucose and lactate levels, pH, and oxygen are monitored automatically. Manual direct examination is necessary to determine cell health including population density, viability, mitotic index, and cell debris (with encapsulated cells samples are also taken periodically to determine cell health).

Flow rates of exhaust gases, unlike fermenters, are too low for meaningful analyses.

3.2.5 Recovery of Product

With cell culture methods the product is secreted so lysis is not necessary. Products retained in microcapsules are recovered by homogenization of capsules after washing to remove medium and small molecular weight components.

3.2.6 Analyzing the Technology

Because cell culture techniques are not as simple or established as those of fermentation there is a much greater need for analysis of the available or developing technology. Although many systems are available few are well established or optimized. There is little reason to expect the economics of one immobilization method to be much better than another with a given cell line and medium.

The advantage of a specific system is often expressed in terms of total cell density attainable. This is obviously a contributing factor in successful culture but it is the total viable count that is the most important parameter; therefore, new methods of quantifying the available systems must be developed in order to extol their virtues.

The system selected should be able to adapt perfusion methodology as this technique is rapidly expanding and if correctly applied may improve the economics of the process.

Because cell culture technology is so complex it is unlikely to become the method of choice for recombinant protein production, especially if genetic engineering can advance to allow all recombinant proteins to be produced in microorganisms. However, necessary use of the method will result eventually in more defined and easier operating criteria.

3.3 In-Vivo Production of Monoclonal Antibodies

The in-vivo production of MAbs involves injecting hybridoma cells of the required line into the peritoneal cavity of a histocompatible host (hence the impossibility of producing in-vivo human hybridomas for general therapeutic use) where they grow and

divide forming tumors. The antibody is produced in tumor ascites (five to fifteen ml per mouse) which are collected for downstream processing. Ascites production can be improved by injecting 0.5 ml pristane into the peritoneal cavity one week prior to the injection of cells.

In-vivo production is the conventional method and has been used extensively. Therefore for applications where purity is not critical and only small amounts of antibody are required, in-vivo production is usually preferred. With the advances in MAb applications larger amounts than can be feasibly produced by mice are being required. For example, a kilogram of antibody that could be produced in a large scale reactor would, in theory, require 20,000 mice if made by the ascites route.

Production in-vivo is considered to produce much better yields than in-vitro probably because of the optimal incubation conditions. However, these conditions are variable, not defined, and are difficult to control. It is probably only a question of time before development enables in-vitro yields to match those of in-vivo.

4.0 Purification

Many of the established methods for protein purification have applications in purification of recombinant proteins or monoclonal antibodies. However, the relative novelty of these materials requires more detailed study of purification methodology because of the nature of potential contaminants.

The extent of purification required is dependent on the intended use of the product. Drugs and biologics to be administered repeatedly or in high concentrations should be "adequately" pure to prevent the development of undesirable immune or toxic reactions.

Because of the relatively new status of biotechnology many purification schemes may be proprietary. Some companies have

disclosed or published details of novel purification strategies but generally procedures applied are conventional methods.

All purification procedures have common objectives:

1. To obtain the desired degree of purity
2. To obtain sufficient quantity of purified material
3. To preserve activity
4. To achieve efficient and cost effective purification

There are two process stages that can be identified. The first is called bulk purification and incorporates disruption of cells, removal of cell debris, and concentration (decreasing volume). The second is called specific purification and incorporates high resolution methods to obtain the final product.

Initial bulk purification for the removal of cells or cell debris is usually facilitated by centrifugation (filtration is less suitable on a large scale) followed by one or more volume reduction processes often which also serve as primary purification processes.

4.1 Purification by Differential Solubility

4.1.1 Salting Out

Precipitation with salts such as ammonium or sodium sulfate is a common method for reducing volume, and simultaneously provides a modest degree of purification. It is widely used in both recombinant protein and MAb production where excess media is filtered or centrifuged off.

4.1.2 Temperature and pH Alteration

Protein instability at elevated temperature or extreme pH can limit application. By careful heating of extracts some inert protein material may be deactivated, precipitated, and removed without destruction of the desired protein.

4.1.3 Use of Organic Solvents

Danger of refolding and consequent deactivation necessitates low operating temperatures (often below 0°C) for fractionation with organic precipitants. Recovery and reuse of solvent may offset the costs of refrigeration for large scale methods.

4.1.4 Use of Aqueous Two-Phase Systems

This method enables concentration and initial purification from culture media, applicable for labile proteins and those subject to high activity loss. Separation occurs due to the incompatibility between aqueous solutions of two polymers (for example, polyethylene glycol and dextran) or between polymer and an appropriate salt (for example, potassium phosphate). The high water content of such a system (65-90%) allows more gentle partitioning of proteins than do water/organic two-phase systems. Enrichment obtained is usually in the same range as that of conventional precipitation methods, but two or three process steps are often required.

4.1.5 Crystallization

Not widely applied in purification. If applicable, it is best regarded as a specific method of fractionation to be used in the final stages when protein is relatively concentrated.

4.1.6 Reverse Micelle Bioseparation and Cell Permeation

The ability to control the solubilization of proteins in organic solutions using reverse micelles would increase the feasibility of using liquid-liquid extraction processes for protein separation. This is a relatively new development as applied to downstream processing. It has been used mainly with fermentation concentrates although nucleic acids and E. coli cells have been successfully solubilized in reverse micelles.

Another new development is in expanding the permeability of cells to obtain intracellular product without disruption. Processes using non-ionic detergents and chaotropic salts have been described although the procedure is still very much in its infancy.

4.2 Purification by Chromatographic Methods

Disruption, solvent extraction, and purification by solubility differences; as well as solid-liquid separation are primarily concerned with protein recovery with little

purification. Specific purification is usually dependent on chromatography. Classical gel-filtration and ion-exchange columns have been supplemented recently with newer adsorption media including a variety of affinity adsorbants with high degrees of specificity. Need for pure product must be considered against potential yield. Each additional purification step results in an unavoidable loss of product. Powerful affinity separations are applied, when possible, to decrease the number of individual processes in purification.

4.2.1 Non-Specific Adsorption

This method is mainly used for laboratory scale using calcium phosphate gel, hydroxyapatite, gamma-alumina gel, and diatomaceous earth. It appears to have little useful application in large scale purification.

4.2.2 Ion Exchange Chromatography

This is a widely used and established method. Ion exchangers include polysaccharides such as dextran, agarose, and cellulose or inorganic substances such as silica and controlled-pore glass. Problems may occur in scale up due to handling difficulties (pre-swelling and fines removal) and operating restrictions (bed-shrinkage and slow flow rate). New adsorption media are being developed for high flow ion exchange systems and some of these, for example, DEAE (Diethylaminoethyl)and CM (carboxymethyl) Sepharose are more suitable for large scale purification.

Chromatofocusing is a variant of ion exchange column chromatography which offers high resolution obtained by separations based on differing isoelectric points.

4.2.3 Gel Filtration (Molecular Sieve)

Used to concentrate proteins, remove salts, and fractionate. Commercially available media include partially cross-linked dextran (Sephadex), cross-linked granulated polyacrylamide gels (Biogen-P), and granulated agarose gels (Biogen-A and Sepharose) with various fractionation ranges from 50,000 to 40,000,000

daltons. Gels of allyl dextran cross-linked with N,N-methylene bisacrylamide (Sephacryl) are suited to large scale because of their improved rigidity and higher flow rate operation.

4.2.4 Affinity Chromatography

This method does not rely on the general physicochemical properties of the molecule but on the presence of specific biological or chemical functions. Because of its specificity, affinity chromatography has been widely applied in the purification of both recombinant proteins and MAbs. A high degree of purity can be achieved frequently in a single step.

A specific ligand is attached covalently to an insoluble polymer or gel, specifically retained proteins are eluted using a displacement agent or by changing pH or ionic strength. The method can be used directly for protein purification or indirectly for the removal of recognized contaminants. Hydrophilic cellulose derivatives, polystyrene gels, cross-linked dextrans, beaded agarose, glass beads, and polyacrylamide gels have been used as insoluble support matrices.

4.2.4.1 Immunoaffinity Chromatography

Exploits the unique specificity, high affinity, and reversibility of antigen-antibody interactions. Rapid expansion of the technique has occurred with the advent of monoclonal antibodies that can be used to isolate specific proteins or vice versa. Agarose activated with cyanogen bromide is the most common support matrix used for immunoaffinity chromatography; however, antibodies of the appropriate class may be purified on staphylococcal protein A-Sepharose columns. Protein A is more stable than most antibodies, although the FDA has not yet approved this technique for producing therapeutic drugs.

4.2.4.2 Hydrophobic Interaction Chromatography

One of the major problems when processing protein homogenates is the presence of proteolytic enzymes. Cooling and addition of protease inhibitors is generally insufficient to reduce

degradation and can introduce contaminants. It has been reported that much of the proteolytic activity can be adsorbed onto hydrophobic gel media. For proteins which themselves do not bind, hydrophobic interaction chromatography could be considered as an early purification step.

4.2.4.3 Dye-Ligand Chromatography

The use of triazine dyes as pseudo affinity ligands has been applied to large scale protein purification. The dyes are easily coupled to matrices containing hydroxyl groups and avoid the use of cyanogen bromide. Exact mechanisms in binding affinity are poorly understood, but many possible explanations have been documented.

4.2.4.4 Immobilized Metal-Affinity Chromatography (IMAC)

Adsorption occurs by coordination between an immobilized metal ion and an electron donor. In normal IMAC the metal ion is immobilized (usually chelated) on a support and the required protein binds via electron donor groupings resident on its molecular surface. IMAC is not yet widely applied in protein purification.

4.2.4.5 Miscellaneous Ligands

The ligand of choice must be capable of chemical attachment to the matrix, must have specific affinity for the molecule to be purified, and the interaction must be reversible to permit recovery. Apart from the above methods, a wide variety of biological interactions have been exploited to provide appropriate ligands including hormones with receptors and enzymes with substrate, product, inhibitor, coenzyme or allosteric modulator.

4.3 Electric and Magnetic Field Separations and Ultracentrifugation

Free boundary electrophoresis, zone electrophoresis on supports such as paper, starch and cellulose powders, electrophoresis in agarose and acrylamide gels, and isoelectric focusing techniques have all been used in protein purification

although few methods are ideally validated or optimized for large scale commercial processing.

New developments in magnetic labeling techniques have extended the useful range of high gradient magnetic separations into many important areas of biotechnology, but developments for protein purification application are limited.

Ultracentrifugation using a density gradient has some application separating by size, density, and shape of the protein. However, the gravitational fields required to resolve proteins from one another are very large and application is generally restricted to large protein complexes.

A wide range of techniques is available for protein purification, but many have inherent problems on scaleup and need to be modified for production use. Expense of the newly developed media is a limiting factor initially, but can be offset by improvement in purification. Much further work on downstream methods is needed before we even begin to approach single step purification or successful continuous rather than batch processes.

Finally, when considering the purification of proteins for therapeutic use, it is not the method of purification used that is important but rather the validation of the purification process. Documented advantages and disadvantages of purification methods vary greatly, and it appears the processes in use are far from optimized on a large scale.

5.0 In Process Control and Validation

In process control and validation are known to be as relevant in ensuring safety, purity, and potency as tests carried out on the final product. In the case of biotechnology products, strict validation of procedure is essential and it is only by carrying out in process controls that confidence in the results of tests carried out on the final product can be assured.

It is possible that as relevant technologies become more established, various cell lines, microorganisms, vectors, and methods of production will become standardized for certain products. It has been suggested that E. coli, yeast, and mammalian cell lines should be tested and registered. In this way the same cell seed could be used for different products. Similarly, some vectors are established in certain hosts and are possibly more acceptable by regulatory authorities than others. However, even with these procedures it would still be necessary to check the properties of the host cell again, once the vector carrying the cloned gene had been inserted. A data bank of existing hybridoma specificities was established under the joint sponsorship of WHO, IUIS, and CODATA in Washington. Before hybridoma cell lines or recombinant microorganisms are put into production a number of specific quality control tests should be made. As cells may be genetically unstable they should be tested routinely for formation of product, and the protein concentration relative to cell volume should be determined. The monitoring of protein production on a large scale requires analytical methods which can detect changes in viable clones or hosts quickly and accurately. Constant monitoring is essential to ensure a reliable supply of product from a genetically consistent source. Techniques which have been employed include electrophoresis and isoelectric focusing, ELISA assays, fast protein liquid chromatography (Pharmacia), Phastgel (Pharmacia), and Cytoflurograph (ODSI) analysis.

Process validation together with final product testing and extensive in process control assays must be combined to give a high degree of assurance of batch reproducibility and final product purity. Process validation allows one to address purity at levels below the sensitivity of final product tests, and also allows use of sophisticated experimental methods which have high sensitivity but which could be considered to be too cumbersome for use in release testing.

One of the major concerns in using continuous cell lines for production is that biological characteristics of an abnormal cell may be transmitted by residual DNA in the product to a human recipient. The unlikely extreme is the transference of tumor-producing potential. Cell lines carrying known oncogenes should, if possible, be avoided. Only a few oncogene sequences have been characterized.

Indirect methods by validation should be used to determine residual DNA in the final product. DNA from the appropriate source may be labeled and its clearance measured at each step of the purification process. Other procedures for determining potential contamination of this type include the use of DNA probes generated by nick translation.

Concern with the amount of DNA only relates to its potential for biological activity. DNA fragments, even if present in relatively large amounts, may be insignificant if there are too few base pairs to cause transformation. It is also known that there are biological barriers in humans that prevent exogenous DNA inducing effect.

Another method of process validation is to perform a full scale fermentation and purification of "blank" cells. This allows direct measurement, by conventional assays, of the protein levels in the fractions that would have contained the product and thereby supply prior knowledge of potential protein contaminants. Radioimmunoassays and ELISA, using high affinity antibodies raised against host cell lysates or similar subcellular fractions, can be used to detect protein contaminants. The success of these methods depends on the same contaminants being present from batch to batch. The system does not make allowances for unexpected antigens, polypeptides originating from a contaminating micro-organism or expression of new host or expression vector proteins. It is therefore important not only to monitor the characteristics of the process itself, but also to examine the culture for microbial contamination at the end of a production cycle.

In process controls are essential to prevent contamination. Endogenous viral contamination is a potential hazard as the majority of mammalian cells contain genetic information relating to retroviruses which can activate or acquire oncogenes from cells. Retroviruses can be detected in cultures by electron microscopy or reverse transcriptase assay. Normal procedures for purification must be shown to destroy or remove retroviruses. Cases have been documented where, even if substrate contains an endogenous retrovirus, the cell may still be considered acceptable depending on the specifics of the product and how it is manufactured.

Nutrient medium is a potential source of contamination especially if serum additives are used. Media should be appropriately treated and screened to prevent culture contamination. Infection with mycoplasma is a particular problem and elimination of infection is not easy. Identification of mycoplasma may be achieved by microscopic examination or staining with DNA specific fluorescent dye. Prevention of contamination by adventitious agents is of primary importance. Cell lines and MCBs should be screened and strict aseptic procedures should be maintained. Tests for contaminants may be performed at any point in the manufacturing process providing the sample adequately represents the material comprising a bulk lot. It is an advantage to test at a point beyond which reproduction of adventitious agents is not feasible.

All products manufactured for use as injections must be sterile. Protein drugs cannot be terminally sterilized by heat and therefore must be produced by strict aseptic processing. In process testing must include microbial environmental monitoring, environmental filter examination and periodic sterile culture medium processing.

6.0 Characteristics of Protein Drugs and Development of Functionally Relevant Standards

Biological products are considered to be those that cannot be completely characterized by chemical and physical methods alone. Because of the novel techniques used to produce recombinant proteins and MAbs, characterization of the product to ensure safety, purity, and potency should incorporate new and established techniques applicable to the specific product and the method by which it is produced. In order to minimize the possibility of untoward effects in patients, such as immunological sensitization, or to identify molecular modifications likely to cause untoward effects there should be precise characterization of the product by specific biological, immunological, and physicochemical methods.

In theory it is relatively simple to develop adequate standards for any one product but because of the variety of ways that product may be produced: using different expression vectors, hosts, cell lines, and process reagents, it is probably not possible to develop overall standards that incorporate all methods of production. Because of this, there are two sets of operating standards to be applied to the final product. The first set is developed by the manufacturer who must apply approved functional tests, relative to method of production, to ensure standards are maintained. The second set are those tests which can be applied to the final product independently without prior knowledge of production details, but which can assure maintenance of safety identity, strength, quality, and purity (see Section 7 Regulatory and Legal Aspects). In general, the latter set of standards would be incorporated within the former.

Tests of identity, purity, and potency of products usually require the use of approved reference standards. There is an immediate need to develop reference standards for recombinant proteins and MAbs. It is possible that reference standards will

have to be relevant to the method of production because of the differing characteristics of products produced by different methods (for example, lack of or differing glycosylation patterns affecting physicochemical and biological properties; or even, as with methionyl hGH, differing amino acid sequences). House standards should be retained samples of well-characterized bulk lots.

6.1 Identity

The evidence required to establish identity depends on the extent of analytical uniqueness of the product. Generally, existing protein chemistry is applicable and identification should be a relatively simple process. It is purity and potency of these products that are more difficult to establish.

Identity tests can be based on chemical, physical or biological characteristics of a protein and can, therefore, be incorporated into various specific tests for purity and potency. It is an advantage to have a specific identity test that can be performed rapidly during and after manufacture.

It must be recognized that products expressed from naturally occurring genes in foreign host cells may deviate structurally from their natural counterparts. Such deviations may arise either at the genetic or post translational level or during purification. Identification tests should therefore encompass several different properties of the molecule and should be carried out in parallel with a reference standard.

Relative Identity Tests

1. Amino acid composition analysis
2. Partial sequence analysis
3. Amino and carboxyl terminal residue analysis
4. Polyacrylamide gel electrophoresis
5. Isoelectric focusing
6. HPLC
7. Circular dichroism and optical rotary dispersion
8. Immunological reactions

9. Use of standard reagents to determine isotype class and subclass of MAb.

6.2 Purity

Levels of purity required are dependent on type and frequency of use of the product. Purity is a relative term defined by the tests used to monitor purity and is bound by the technical limits of those tests. Impurities in a biological product can be defined as all substances that are not considered to be active drug, known excipients or additives. Impurities may include substances derived from the active entity (by-products or decomposition products), as well as non-related components such as E. coli protein and endotoxin. Impurities may or may not include those proteins that have undergone proteolysis at specific sites in the molecule. The determination of this particular problem must be made on the basis of data that demonstrates whether or not these molecules have the same or similar properties to the intact drug substance. Impurities may be innocuous with no health or safety concern or significant where there is health or safety concern particularly with respect to toxicity or immunogenicity.

6.2.1. Physicochemical Purity

As well as establishing identity of the active protein, chemical purity must be assessed. Due to the possibility of degradation products of active protein by proteolytic enzymes in homogenates, tests to identify lytic products should be applied. If lytic products are present it must be established as to whether contaminating proteases are present or whether the product is autolytic. Particular attention should be paid to sequences at amino and carboxyl terminal regions of product because proteolytic degradation often results in a product with "ragged" ends.

Some proteins have been shown to undergo irreversible clipping (for example t-PA) which as with autolysis may or may not result in loss of biological activity. Limits for maximum number of clips, maximum lytic product content, and minimum content of single chain material may be relevant.

Mutants and formyl-methionine impurities can be detected using tryptic mapping methods; oxidized methionines can be detected by amino acid analysis, tryptic mapping or Edman degradation. Deamination and proteolytic clips may be detected using isoelectric focusing and SDS-PAGE techniques which can also be used to identify any light or heavy chains a hybridoma may secrete. With MAbs it is possible that the desired product may be an immunoglobulin fragment in which case degree of homogeneity must be established.

Separation science should also be applied to detect presence of dimers or aggregates. The formation of dimers can be reducible or non-reducible and can often occur during lyophillization of a product. Dimers in recombinant hGH have been documented at 0.1% of final product.

It is necessary to ensure that protein products have the three-dimensional conformation required for biological activity. Some experience with E. coli has revealed that high levels of intracellular expression of cloned eukaryotic proteins is often accompanied by significant denaturation (for example, unfolding). Tertiary and quaternary structure can be verified by Xray crystallography and circular dichroic spectra; peptide mapping can be used to verify the correct arrangement of disulphide bonds, and SDS-PAGE can be used to discriminate various glycosylation patterns. Verification of the correct folding is of great importance if the product is formed by chimeric fusion of recombinant chains (for example, insulin).

Other important tests that may be applied to ensure physicochemical purity include determination of molecular weight, isoelectric point, and optical rotation.

When proteins are produced by organic synthesis certain chemical impurities are inevitable. Production of complex polypeptides is a multistep process with yields of less than 100% at each step. Even with yields of 99% at each step synthesis of a

32 amino acid peptide gives a final product of which only approximately 72% is required protein, the remainder being all combinations of single and multiple amino acid deletions along the chain. This results in purification difficulties because of the necessary separation of many very similar entities.

6.2.2 Immunological Purity

Bioactive proteins or peptides derived from cells used in culture, from host microorganisms or from contaminating bacteria may be clinically significant if residual amounts are present in the final product. Residual proteins may be recognized as antigens by the patient and stimulate immune response. They may also cause reactions with pre-existing immunity which could result in tissue damage by various allergenic mechanisms.

Potential host cell protein contaminants can be detected by "blank" fermentations (see Section 5). Fractions of a blank fermentation for hGH production in E. coli indicated that contamination was 120 ppm with two column purification steps left and little protein remaining to complete the process. Using a variety of methods including immunoassay, process validation and final product analysis methionyl hGH purified from E. coli has been obtained with a purity of greater than 99.9%. Similar blank fermentations during validation of insulin production in E. coli showed only very small quantities of peptide contaminants and these were shown to be non-antigenic except in complete Freund's adjuvant.

The presence of protein contaminants may not be an important issue. Humans already have circulating antibodies to E. coli proteins; animal insulins which have been used in therapy have potential hormone or protein contaminants which can be highly immunogenic, and various vaccines used clinically have contained bovine serum protein up to a concentration of 1 µg per dose without causing allergic reactions.

One guideline for setting a maximum limit for protein or peptide contamination has been based on a very potent immunogen, fetal calf serum, which has previously contaminated a number of biologics. Experience has shown that 100 ppm contaminant foreign protein has not resulted in unsafe products. However, acceptable limits will vary depending on proposed method and frequency of administration, source and potential immunogenicity of protein contaminants and whether the limits are set for total protein contaminants or specific protein contaminants. Biosynthetic human insulin has been documented as having an E. coli peptide content of less than 4 ppm. A potential new product produced from mammalian cells under consideration by the FDA has a total foreign protein content of less than 1000 ppm for a once daily dosage.

In settling the requirements for the absence of foreign proteins, evaluations in animal models should be carried out to determine at which level a certain protein contaminant might cause adverse reaction; however, the clinical significance of protein contaminants is largely unknown before the product is clinically tested in humans, and it should be recommended that patients receiving these products should be routinely monitored for antibody formation and allergic manifestation, especially if the product is to be administered in large or repeated doses. For MAb produced by in-vivo methods, the assessment is further complicated by the fact that the murine origin products themselves may be immunogenic.

Products administered repeatedly or in large doses should be assayed for trace antigenic constituents. Tests such as Western blots, radioimmunoassays and ELISA using high affinity antibodies raised against the product's host cell lysates, appropriate subcellular fractions, and culture medium constituents should be used to detect contaminating antigens. Because the detection of antigens will be limited by the specificity and sensitivity of the antisera used, these immunoassays should be complemented by use of

silver stain analysis of SDS-PAGE, reverse phase and size exclusion HPLC.

Endotoxins or pyrogens cannot be detected in protein assays. The importance of bacterial endotoxin is well recognized, specifically the endotoxins secreted by E. coli. A fairly large number of continuous cell lines have also been found to produce cytokines (for example, Interleukin 2) which could have direct biological effect if retained in the product. Pyrogens are fever causing materials, often of low molecular weight (for example, certain peptides and liposaccharides from gram negative microorganisms). Humans are known to be extremely sensitive to pyrogens and unfortunately some substances are pyrogenic only in humans.

The removal of pyrogens or endotoxins can be carried out by selective adsorption on electrostatic filters, precipitation with alcohol, and immunoadsorption (using an immobilized antibody to a known pyrogenic substance). Pyrogens that result from extraneous microorganisms may be prevented by using sterile processing conditions and pyrogen free materials and equipment.

Tests for pyrogens are designed to limit to an acceptable level the risks of febrile reaction in the patient to the administration, by injection, of the product concerned. Common tests for pyrogens include the USP rabbit pyrogen test, reliable for detecting nanogram quantities of toxins but affected by the variability in rabbit breeds, the environment of pyrogen testing conditions and the well known ability of rabbits to develop tolerance. The Limulus Amebocyte Lysate (LAL) assay for detection of subnanogram quantities of toxins has been a major advance and many manufacturers are using it alongside the USP test; however, it detects only endotoxins so several non-endotoxin pyrogens are not detected in the LAL assay. Unfortunately neither of these tests exactly parallels the fever response in humans and certain biologicals have been pyrogenic in humans despite having passed

both tests. One new method for detecting exogenous pyrogenic material is the Human Leukocytic Pyrogen Assay. The ability of exogenous pyrogenic materials to stimulate leukocytic pyrogen (LP) release from human cells provides a more specific assay to detect pyrogenic materials. Early lots of methionyl hGH which were non-pyrogenic in rabbits at 2.5 mg/kg and contained less than 0.4 ng/mg of LAL positive material, did however produce a pyrogenic reaction in phase 1 trials at a dose of 0.1 mg/kg. The human LP assay was subsequently used to select the purification scheme and formulation which yielded a non-pyrogenic preparation. One secondary advantage of the LP assay is that it can differentiate between a pharmacological product which is intrinsically pyrogenic and one which is contaminated with unknown exogenous pyrogen. This could be inherently useful if recombinant DNA technology advances to production of completely novel proteins.

Results of endotoxin tests are usually documented in terms of endotoxin units (EU) per vial or mg product. Depending on the frequency and size of dose a significant amount has been documented as greater than 5 EU/kg patient.

The USP rabbit test is an established and widely used method of pyrogen determination. With the introduction of the new assay methods, manufacturers are still using the USP test either alone or in conjunction with new tests. It appears that certain pyrogen endotoxin tests may be applicable in certain areas and the use of multiple tests in many cases may be unnecessary. There is a need for validation of pyrogen testing. The advantages and disadvantages of each test need to be assessed so that only reliable methods are used to detect pyrogen contamination.

6.2.3 Purity in Terms of Absence of Adventitious Agents

(see also section 5 In Process Control and Validation and section 6.2.5 Toxicity)

Adventitious agents are living, viable or activatable biological agents inadvertently present in the process or product.

In a bacterial host fermentation likely contaminants include non-host bacteria and bacteriophage. In a mammalian cell culture contaminants include non-host mammalian cells, bacteria, fungi, mycoplasma, endogenous and exogenous viruses.

Adventitious agents can compromise cell function prior to expression. They can cause potential degradation of product after expression and apart from causing infection may be a source of extraneous material if present in the final product.

Potential contamination by adventitious agents enhances the need for strict in process control. Tests for these agents should be applied to bulk lot or final product but prevention of contamination during production is the major necessity.

Tests for Adventitious Agents

1. Non-host bacteria -- plate on several selective media
2. Non-host mammalian cells -- manufacturers working cell bank (MWCB) procedures
3. Bacteriophage -- plaques on bacterial lawn
4. Bacteria and fungi -- sterility tests
5. Mycoplasma -- culture tests (agar and broth), staining with DNA specific fluorescent dye
6. Endogenous and exogenous viruses -- reverse transcriptase assay, nucleic acid estimations, hemadsorption, viral susceptibility

Tests used should be appropriate to the cell substrate and culture conditions employed. Electron microscopy is also a valuable tool in evaluating adventitious agent contamination.

As opposed to other potential contaminants where limits can be set for maximum contaminant content, products must be free from microbial, viral, mycoplasma, and fungal contamination.

6.2.4 Sterility

Any product for parenteral use must be sterile. Protein products cannot be terminally heat sterilized in final container and are therefore produced by strict aseptic processing. Absolute

sterility (complete absence of viable microorganisms) cannot be practically demonstrated without complete destruction of every finished article; therefore, both bulk material and a specified appropriate number of finished product units should be tested for sterility usually using fluid thioglycollate medium or soybean-casein digest medium (USP methods).

6.2.5 Toxicity

Toxicity from a biotechnology product can result from the presence of many components, of primary importance is residual DNA (see section 5). Other causes are adventitious agents, endogenous or exogenous proteins, lipids, carbohydrates, or various other contaminants and residual process reagents (antibiotics are used in fermentation during the production of Intron A and Roferon A but both manufacturers state that the antibiotic is not detectable in the final product).

Although process validation and control must be used to eliminate or reduce residual DNA in the final product, tests on final containers should be done using the most sensitive techniques available. Dye binding fluorescence enhancement assays and DNA hybridization analysis of immobilized nucleic acid on nitrocellulose filters using appropriate probes such as nick translated host cell and vector DNA may be used. Depending on the source of residual DNA and the potential use of the product, tests on final containers may be important. Limits for the content of residual DNA, often stated at 10 pg/dose, may however be irrelevant. What has to be established is the form that the DNA is in and its potential for inducing biological effect. Theoretical concerns regarding transforming DNA derived from the cell substrate will be minimized by the general reduction or elimination of contaminating nucleic acid.

Apart from contaminants or adjuvants in the product the inherent toxicity of the active entity must be established. Using recombinant procedures proteins may be modified to enhance their

desired properties or diminish undesirable properties. There is also the potential for production of completely novel proteins. As stated in section 2.3.3, any substance that is not a natural constituent of the human body may be antigenic and may also cause unknown and possibly adverse biological effects. The use of such a product depends on careful assessment of its new benefits compared to the risks identifiable in preclinical and clinical evaluation.

The role of toxicity testing in evaluating the safety of a product is to allow establishment of a risk to benefit ratio. This cannot be achieved accurately but a satisfactory margin of safety should be assessed by taking into account the nature of the biological product, its properties, and its effect upon the patient. Safety evaluation can be an expensive and time consuming phase of development, the degree of study required varying considerably depending on the use of the product.

A general safety test for the detection of extraneous toxic contaminants must be performed on all biological products intended for human use. This test however, is not designed to identify intrinsic toxicity of the product. Specific pre-clinical testing needs such as carcinogenicity, teratogenicity, and effects on fertility may be necessary using appropriate in-vitro and in-vivo animal tests.

As with most injectable substances the toxicity of MAb products is ordinarily evaluated in laboratory animals: however, traditional toxicology test procedures such as LD50 determinations are unlikely to provide information which will be relevant to the uses proposed for most MAbs. No single animal system has been described which is sufficiently useful to be considered a required universal test; nevertheless, preclinical animal tests appropriate to the intended use of the product are strongly encouraged by the FDA. A description of these non-clinical studies concerning the

drug's pharmacological and toxicological effects is essential for application to start human clinical trials.

MAbs are homogenous populations of immunoglobulin molecules. In some instances the antigenic determinant or a structurally similar epitope may be expressed on human tissues or cells other than the intended target. Hence undesirable cross-reactions may occur. Similarly, endogenous proteins generally have more than one action in the body. These actions must, if possible, be identified so that potential adverse effects can be assessed.

In-vitro or in-vivo animal toxicity tests can only supply limited information. Many toxic effects of a product will unfortunately not be identifiable in pre-clinical testing. Once a product has undergone sufficient toxicity tests to warrant further investigations, acute toxicity tests as single doses in normal human subjects, and then long term safety studies are carried out under strict regulatory control (see section 7.2 Regulatory Agencies).

It is apparent that some of the procedures mentioned will be useful in establishing only the identity of a product while others will provide information on both identity and purity, yet still others will provide evidence of purity alone. It is unlikely that all tests will be needed for each batch of the product. Some tests will be required only to establish the validity or acceptability of a process, others may be performed on a limited series of batches in order to provide data on consistency of the product and its production. Modification or elimination of certain existing tests or the introduction of new tests and control procedures will inevitably occur as knowledge of these products accumulates. There is a need for validation of existing test procedures for specific products. At this early stage there may be a tendency to apply as many tests as possible to ensure purity of a product, but relevance is the key word for new testing methods.

6.3 Potency

The essence of potency is that of a selected response induced by a product as measured directly or indirectly by comparison with a standard reference preparation.

The majority of proteins being produced by rDNA techniques are those that have a particular biological activity of some physiological or immunological significance. There are therefore two different methods by which they can be assayed: either as chemical substances or by utilization of a biological response.

6.3.1. In-Vitro Analytical Assays

Analytical assays utilize the procedures of quantitative analytical chemistry and immunochemistry to characterize and assay the number of molecules that have the requisite physicochemical or antigenic characteristics of the protein.

6.3.1.1 Physicochemical Assays for Protein Content

Physicochemical assays can provide information on identity and purity as well as protein content. They have the advantages of precision, accuracy, selectivity, speed, and convenience and can also be used as stability indicators. However, they do not measure the amount of functional product.

Small modifications (for example, change of a single amino acid) may totally change the biological activity of a product (oxidation of the critical methionine residue of parathyroid hormone decreases biological activity to 10% or less of the original hormone). This problem would be detected by physicochemical assay methods, but even intact molecules recorded by analytical assays may not have full biological activity depending on the configuration of the product in the sample. Procedures used for some physicochemical assays are carried out under toxic, non-physiological conditions and in some cases the procedures themselves may alter the conformation of the protein.

Protein Content Assays (physicochemical)

1. Kjeldahl nitrogen
2. Lowry/Biuret -- colorimetric methods

3. UV spectrophotometry -- extinction coefficient
4. Dry weight analysis
5. Quantitative amino acid analysis
6. Quantitative HPLC analysis

6.3.1.2 Receptor and Ligand Assays

The most widely used analytical assays are immunoassays, predominantly radioimmunoassay and radioreceptor assays. All such assays have the same prime components: analyte, standard, a binding protein, and a method of signal detection and amplification.

Radioimmunoassays and immunoradiometric assays use labeled analyte or binding reagent. Various immunoreactive sites on the analyte can be used depending on the antibody. Specificity characteristics vary with antiserum used.

Radioreceptor assays with labeled analyte or specific receptor utilize target cell receptor-sites so the biologically active site on each protein is constant and the structure and specificity of receptor sites on each different target cell or tissue are relatively constant.

Detection is usually by radioactive isotope disintegration and amplification by photomultipliers. These assays are simple, rapid, highly precise, are readily validated, and suitable for large numbers of samples. When properly designed they can, in certain cases, reflect biological activity but they are not assays measuring functional product.

The implicit premise in the use of immunoassays for this type of assay is that antigenic determinants are present only as intact protein molecules and that these molecules have biological activity. This is not always true. Many enzymes, for example, those that possess allosteric properties, can occur in various forms ranging from fully active to inactive. Fully active polymers may depolymerize into less active trimers, dimers or inactive monomers. Immunoassay may not distinguish between full polymer and monomer.

Analytical assays may detect non-functional molecules but may also confuse closely approximated molecules. The fact that a molecular species binds to a receptor does not necessarily mean it will exert the required biological effect in-vivo. Molecules that closely resemble the protein chemically, may bind and inhibit the action of the original protein.

6.3.2 Bioassays

Bioassays are methods of determining potency in a clinically meaningful way. They measure potency by quantifying response of a biological matrix to a specific standard. The matrix may be a whole animal, isolated or in-vivo cells, organs or enzyme systems. Detection and amplification of bioassay is by measurement of a specific biological response. It is insufficient to specify that the product shows some qualitative response, this must be quantified to serve as an assay. Qualitative response on the other hand may serve as a test of identity or as a confirmatory test in conjunction with a validated chemical assay.

The design of bioassay depends on the specific biological function to be measured. Some examples are given in his review but the potential for design is very broad. Many proteins have multiple biological functions: hGH promotes the growth of skeletal and soft tissue and influences the metabolism of carbohydrate, fat, and protein. These various biological characteristics have been used to set up in-vitro and in-vivo bioassays for growth hormone activity. The somatotropic property of hGH in hypophysectomized rats is determined from weight gain and widening of epiphyseal cartilage. Other tests measure the insulin and lactogenic properties.

6.3.2.1 In-Vitro Bioassay

Examples of in-vitro bioassays for hGH:

1. ^{14}C glucose utilization, conversion to $^{14}CO_2$ using hypophysectomized rat epididymal fat body

2. ^{3}H leucine incorporation into protein using hypophysectomized rat diaphragm
3. Induction of N-acetyl lactosamine synthetase using cell culture preparations of mouse mammary glands

Cytochemical bioassays for peptide hormones and some biologically active immunoglobulins are becoming more widely applied as they have a greater sensitivity than radioimmunoassays. Initially, cytochemical bioassays were performed on segments cut from the target organ of one animal, therefore removing the problem of interanimal variation. Today they are generally section bioassays using sections sufficiently thick to encompass whole cells. The target cells in these specifically prepared sections respond to the active entity, in theory, with the same sensitivity as when the protein is presented in-vivo.

Cytochemical bioassays can also be used to detect similar molecules acting as inhibitors by recovery experiments. A known concentration of a reference preparation of known potency is added to the sample. In the absence of any inhibitory influence the biological activity of the reference sample will be fully expressed.

As stated previously in-vitro bioassays can be extremely diverse because they are designed according to a protein's biological function. For example, t-PA is an enzyme having clot lysis as a biological action. This action can be utilized to design an in-vitro bioassay as long as reagents are standardized. It would therefore be necessary to standardize the clot to be lysed, to use reference fibrinogen and reference t-PA. In this type of bioassay the potency could be determined relative to the time taken to dissolve the clot.

Antiviral and antiproliferative effects of IFN can be used to design in-vitro potency assays. Generally potency testing of IFN uses in-vitro cell culture methods with virus interference assays. Protein determination and radio and enzyme-linked immunoassays

have also been used but the extent to which results correlate with biological activity need to be established on an individual basis. A variety of cell lines are sensitive to IFN effects when challenged with viruses; human cell lines are preferred for correlation reasons. Viruses used include vesicular stomatitis virus, encephalomyocarditis virus, and sindbis virus. Standardization of the amount of virus used is essential and the extent to which variability of virus inoculum affects variability of assay results must be determined.

Generally, in-vitro bioassays have the advantage that they measure a biological function. They are less expensive and slightly more precise than in-vivo bioassays. They can be automated but generally require multiple samples and are difficult to troubleshoot.

6.3.2.2 In-Vivo Bioassays -- Animal

In-vivo bioassay systems are relatively few and are generally well described in compendia. The measured response again generally reflects the desired effect of the analyte.

Specific examples include:

1. Bioassays for insulin -- rabbit blood sugar test
   -- mouse blood sugar test
2. Bioassays for hGH -- ten day weight gain in hypophysectomized rats
   -- changes in length of tibia (rats)

Bacterially produced vaccines must be shown to give rise to virus neutralizing antibodies to demonstrate antigenic activity relating to potency. The mice potency test is often used as a standard assay reacting in-situ; after a period of time mice are bled, sera collected, and antibody titers determined by radio-immunoassay.

The majority of in-vivo potency tests are done on subprimates: mice, rats, and rabbits. Generally, potency

estimations cannot be extrapolated from one species to another because of evolutionary alterations in structural requirements of receptors, metabolic rates, and variations in pharmacokinetic parameters. Potency estimations in primates may be done to provide baseline information that may be more applicable to in-vivo human use but there are some major disadvantages in using in-vivo bioassay systems for potency estimation.

In-Vivo Animal Bioassays

1. Expensive due to the large number of animals required to overcome interanimal variation. The cost is increased because of the need for statistical support
2. Sometimes insensitive due to dilution of protein in body fluids
3. Technique dependent
4. Slow in operation, generally require a number of days to complete
5. Many variables need to be standardized

In general there is a great social pressure to replace in-vivo animal studies with relevant in-vitro studies. There are advantages and disadvantages as stated for all methods of potency determination, but an assay measuring biological response should be applied at some stage to ensure required activity.

In-vitro bioassays have advantages over both in-vivo assays in animals and analytical techniques. If a suitable in-vitro bioassay can be designed it will probably be less expensive, more sensitive, and more precise than a whole animal study and will possibly have the advantage of directly measuring potency.

6.3.2.3 In-Vivo Bioassay -- Human

Once basic dose response and toxicity levels have been established, Phase I clinical trials in normal human subjects are carried out. These tests are for acute toxicity and to establish a dose response relationship. Physiological studies as well as potency estimates must be carried out to determine how the

biological activity of the protein may be affected by the metabolic activity or route of administration. Phase I clinical trials are generally single dose blood or urine studies. For example, hGH activity in man is determined by monitoring retention of nitrogen, potassium, sodium, and chloride or by changes in levels of plasma free amino acids, free fatty acids, and urea. Activity is then quantitatively related to concentration and dose response relationships can be established.

6.3.3 Potency Estimations for Hybridoma Products

Protein content assays may be used as discussed previously. The binding characteristics of a monoclonal antibody product, used either unconjugated or to deliver therapeutic drugs, must be established. The ability to bind strongly and specifically to a target cell or molecule determines potency in-vivo.

There are basically four parameters that characterize a monoclonal antibody product:

1. Specific activity
2. Immunologic specificity
3. Binding affinity
4. Antibody content

The ability to bind strongly and specifically is generally determined during screening procedures but total antibody content should be determined on the final product.

6.4 Stability

During the past twenty years or so the pharmaceutical industry and regulatory agencies have gained considerable experience and competency in dealing with stability problems in estimating realistic shelf lives. Such documents as Fung's authoritative chapter (chapter seven in "Modern Pharmaceutics" 1979) on the stability of pharmaceuticals or the FDA Draft Guidelines on expiration dating (1984) exemplify the depth and breadth of knowledge in the area of drug product stability.

It might be tempting to assume that the formulation of reasonably stable pharmaceutical products and the assignment of reliable shelf lives to such products is now a matter of routine. Such a thesis is of doubtful validity for conventional (non-macromolecular) drugs; it is even more suspect for protein drugs. Most experts in the field of drug product stability would probably agree that there are questions concerning the stability of protein drugs and devices to which, at present, the answers are somewhat obscure. There are several reasons for this uncertainty. One of the major problems which presently confronts us is related to our lack of knowledge of what group or groups in a macromolecule are essential and what are not for efficacy or toxicity. In the absence of knowledge it is often difficult to state with assurance whether or not any given single assay method is truly stability indicating. We know that proteins are susceptible to a variety of chemical and physical changes which may modify primary, secondary or tertiary structure. In some cases these changes can quantify relatively easily in others the task is difficult.

Adding to the complexity of the task of preparing stable products is the fact that although a number of companies have recently successfully developed stabilized formulations of proteins such companies have not unnaturally displayed considerable reluctance to publish their data. No doubt, in time such stabilization techniques will become generally known but at present there is considerable replication of effort by different companies all investigating an essentially common problem.

Perhaps, the most pressing problem, which now faces those wishing to market a new macromolecular drug or device, is the establishment of the initial shelf life. FDA has for a number of years allowed - within certain limits - the use of accelerated stability testing techniques based implicitly or explicitly on use of the Arrhenius equation for the initial definition of the shelf

life of some micromolecular pharmaceuticals. It has been pointed out that even for micromolecular products there are cases when it is not valid to use an Arrhenius type approach (Wolfe and Worthington, (1976)). In particular, at present there is a paucity of reliable experimental or theoretical data which would support the use of an Arrhenius type approach for the prediction of physical degradation (Rhodes, C. T., (1984)). It is probable that a complete resolution of the problem of assigning reasonable shelf lives to protein drugs and devices must await the testing of a variety of evaluation methods on different products and publication of such data. However, even now there are a number of useful papers which address regulatory, theoretical or practical aspects of this topic (references section 6).

7.0 Regulatory and Legal Aspects of Macromolecular Drugs and Devices

7.1 Compendial

The United States Pharmacopeial Convention's (USPC) mission is to establish drug names and to publish official drug standards for identity, strength, quality, and purity. The revision process is open to input from all interested parties. The standards are set by the USP General Committee of revision composed of elected volunteers from the medical and scientific communities, from government, industry, and academia. Pharmacopeial scope extends to all drugs including macromolecular drugs.

A USP monograph is a quality standard. Any individual sample of a USP recognized drug when tested by the compendial tests conforms to the monograph specifications. This does not mean that every batch of the monographed product has to be submitted to compendial testing prior to release.

USP tests and assays are the only referee tests and assays in case of dispute in terms of compendial appliance. They are derived using knowledge of the expected contaminants based on classical drug manufacturing processes. If a macromolecular drug

is produced using novel biotechnological processes, compliance of the final product to existing compendial requirements for identity, strength, purity, and quality will obviously be insufficient. Additional tests for purity, identity, quality, and safety will be needed. These additional tests and assays will have to be developed on a drug by drug basis until the establishment of an extensive track record in the field allows quality, purity, strength, and safety generalizations for the products.

On a more practical note, USP General Notice, makes provisions for alternative methods for compendial tests and assays. These alternative methods must be validated to show at least equivalent suitability for determining compendial compliance.

The USP addresses the use of reference standards. A USP Reference Standard is a reference product that has been tested collaboratively and approved by the USP Reference Standard Committee only on the basis of suitability for use in official USP assays and tests. In general, the USP Reference Standard is provided by the manufacturer, say of a macromolecular drug; it is subjected to collaborative testing by at least three laboratories to establish its labeled potency and its biochemical characteristics. The evaluation includes testing by all the relevant compendial tests and assays as well as by additional testing and assessment of stability data. USP Reference Standards do not have an expiration date since monitoring of the stability of the standards and publication of current listings are an integral part of the USP Standards Program.

A USP Reference Standard for a macromolecular drug would assure the reproducibility of results in compendial testing in spite of the instability of reagents, supplies and systems. It would also minimize the need for detailed and comprehensive description for the preparation of standards by users, and reduce

the variability in testing between operators, equipment, and replicate assays.

7.2. Regulatory Agencies

As the scale up from laboratory setting to large scale manufacturing of protein drugs and devices made by rDNA or hybridoma techniques continues, the question of regulatory control over methods of manufacture and final product becomes increasingly important. Companies looking towards marketing products are obviously interested in the extent and form of existing authority. Obtaining regulatory clearance to manufacture and market products often involves millions of dollars and enormous expenditures of resources. Regulatory hurdles can result in long delays in delivery of useful products and can drive up consumer costs. It is therefore necessary to be fully aware of the regulatory methods that apply.

Regulation of the biotechnology products discussed in this review fall under the statutory authority of four federal agencies: The Food and Drug Administration (FDA), The Environmental Protection Agency (EPA), The Occupational Safety and Health Administration (OSHA), and The United States Department of Agriculture (USDA).

These agencies have extensive powers to regulate the manufacture and distribution of products under appropriate Acts of Congress. There are also research policies and guidelines that have been published by various bodies such as the National Institutes of Health (NIH), National Science Foundation (NSF), EPA, FDA, and USDA.

All the regulatory agencies mentioned have existing policies applying to already established drugs and devices. When new methods of producing drugs and devices evolve, existing regulations have to be analyzed to establish whether they provide sufficient control or whether new regulations should be formed. Questions to be asked include: How revolutionary is the

technology? Is it unique and novel? Does it result from the synergism of developments in several complementary disciplines, or is it simply a new application of an existing technology perhaps adapted from another discipline?

It is necessary to define how these products should be categorized in order to decide which agencies are relevant in their regulation and to ensure that sufficient expertise to evaluate regulatory requirements exists within these agencies.

The sciences of rDNA and hybridoma are not revolutionary but certainly their application to development of drugs is. Jurisdiction over the various products is logically being determined by their potential use as has been the case for traditional products.

Because the biotechnology industry is still evolving, the formation of relevant regulations is still under discussion. At the time of editing this paper the latest guide to Federal regulations and policies, Part II. Office of Science and Technology Policy "coordinated framework for regulation of biotechnology; announcement of policy and notice for public comment" has just been released. Certain concepts are new to this policy and will be the subject of rulemaking following public comment.

7.2.1 Food and Drug Administration (FDA)

Congress has provided FDA authority to regulate certain products regardless of how they are manufactured. The FDA is responsible for assuring that each product under their jurisdiction meets the required standards of potency, safety, purity, and efficacy. When addressing products produced by rDNA or hybridoma technology, obviously certain established standards apply but new standards have to be developed alongside the technology's new application.

Products under the jurisdiction of the FDA are regulated by: The Center for Human Drugs and Biologics, the Center for

Veterinary Medicine (veterinary drugs), and the Center for Medical Devices. These Centers, in turn, have general policies for approval of new drugs, biologics for human use, animal drugs, and medical devices.

A new drug is a drug not generally recognized by qualified scientific experts as safe and effective for the proposed use. A biological product is any virus, therapeutic serum, toxin, antitoxin, vaccine, blood, blood component or derivative, allergenic product or analogous product applicable to the prevention, treatment or cure of diseases or injuries of man. A medical device is a health care product that does not achieve any of its principal intended purposes by chemical action in or on the body, or by being metabolized. (Federal Register, Vol. 51, No. 123. June 26, 1986.)

The marketing of new drugs and biologics requires prior approval of an appropriate new drug application (NDA), biological product license or new animal drug application (NADA). For new medical devices, including diagnostics, either a premarket approval application (PMA) or a reclassification petition is required. If the device is determined to be equivalent to an already marketed device a premarket notification under section 510(K) of the Federal Food and Drug Cosmetic Act is required. Biological products must be approved by FDA prior to marketing as required by section 351 of the Public Health and Safety Act. Unapproved biological products are regulated under the same regulations as new drugs during the investigatory phase. Prior to licensing separate licenses are issued for the establishment and the product. Manufacturers of new drugs and biologics must also operate in compliance with current good manufacturing regulations.

There are many scientific issues that have been raised over FDA regulation of rDNA and hybridoma products. Many questions have arisen concerning the need for new or supplemental marketing applications for products that are identical to products produced

by more conventional methods. However, the use of, for example, rDNA has the potential to lead to new structural features in the product, result in product micro-heterogeneity or introduce new contaminants. Despite some experience with drugs derived from microorganisms such as vaccines, antibodies, and enzymes there is little experience with such substances employed as parenterals in humans with continuous administration.

Approval of the product application is also approval of the manufacturing technique and quality control procedures. Until the technologies are more established and uniform, each application must be considered on a case by case basis.

New animal drugs under the jurisdiction of the FDA are subject to similar procedures as new drugs for human use, although investigational new animal drug regulations do not require advance agency approval for clinical investigations. FDA's Center for Veterinary Medicine has also determined that when the new substance produced by biotechnology is identical, or virtually identical, to an approved substance produced by conventional technology, only a supplemental application is necessary if the two products are produced by the same manufacturer.

Medical devices for human use are regulated by the requirements of the Act as amended by the Medical Device Amendments of 1976. The Act establishes three classes of devices depending on the level of regulatory control required.

All premarketing approvals of FDA regulated products are subject to the requirements of the National Environmental Policy Act (NEPA), as defined by the Council on Environmental Quality Regulations (40 CFR parts 1500-1508), and as further described by FDA's NEPA Implementing Procedure (21 CFR part 25).

To provide guidelines for prospective manufacturers of drugs and biological products by rDNA and hybridoma technology the FDA has developed a series of publications as follows:

1. "Points to consider in the production and testing of new drugs and biologicals produced by rDNA technology." (April 1985)

2. "Points to consider in the manufacture of monoclonal antibody products for human use." (July 1983)

3. "Points to consider in the manufacture of in-vitro monoclonal antibody products subject to licensure." (June 1983)

4. "Points to consider in the characterization of cell lines used to produce biologicals." (June 1984)

5. "Points to consider in the production and testing of interferon intended for investigational use in humans." (1983)

The FDA also has published guidelines on general principles of process validation, (March 1986), and sterile drug products produced by aseptic processing, (Jan. 1985).

7.2.2 Environmental Protection Agency (EPA)

A number of statutes passed by Congress are directed towards protection of the environment. These include the NEPA and the Toxic Substances Control Act (TSCA). Specifically, EPA reviews chemical substances under the TSCA. Microorganisms and their rDNA molecules are designated as chemical substances but the EPA has stipulated that chemical substances used as drugs, and medical devices, and microorganisms that are used to produce, or used as drugs and medical devices will not be reviewed under TSCA.

7.2.3 United States Department of Agriculture (USDA)

The USDA is responsible for regulation of veterinary biological products under the authority of the Virus, Serum, Toxin Act of 1913. Questions as to whether a product is an animal biologic, or an animal drug, are referred to a standing committee of representatives from FDA and USDA.

Veterinary biologics are defined in governing regulations (9 CFR 101.2 (W)), licensing provisions for veterinary biological products and establishments are found in part 102 of USDA regulations (9 CFR part 102).

The USDA states that veterinary biologic products prepared using modern biotechnology procedures will be treated similarly to products prepared by conventional techniques. The department

strongly recommends that all applicants establish institutional biosafety committees and intends to propose guidelines which specifically relate to veterinary biologics.

7.2.4 Occupational Safety and Health Administration (OSHA)

OSHA has received its responsibility under the Occupational Safety and Health Act of 1970 as it relates to the protection of workers in biotechnology. Section eight of the act authorizes OSHA to inspect workplaces relating to biotechnology. Section five concerns the responsibilities of the employer. No new regulations or new applications of existing regulations have been developed to apply specifically to biotechnology industry at this time.

7.2.5 National Institutes of Health (NIH)

The primary role of NIH in relation to biotechnology has been the funding of biomedical research. In addition the NIH was the first recognized group to address the safety of rDNA research. The role of NIH is described in detail in the NIH Guidelines for Research Involving Recombinant DNA Molecules. A complete republication of these guidelines appears in the Federal Register May 7, 1986 (51 FR 16958).

The guidelines are applicable to all rDNA research within the United States which is conducted at, or sponsored by an institution that receives support from NIH.

Part VI of the guidelines, added in 1980, entitled "voluntary compliance" states that individuals, corporations, and institutions not otherwise covered by the guidelines are encouraged to do so. Many companies including those commercially producing protein drugs and devices using rDNA techniques have registered with NIH and the guidelines have evolved to incorporate large scale manufacturing processes.

In conclusion, no dramatic new regulations have been introduced for biotechnology products as discussed in this review. The administrative review of procedures and products has been

based on existing regulations and the intended use of each product, on a case by case basis. The unlimited number of products that may result from application of rDNA and hybridoma technology make it impossible, at this time, to define all requirements in specific terms. Each product and method of manufacture has to be evaluated individually to determine what will be necessary to ensure its safety, purity, potency, and efficacy.

The Office of Science and Technology policy states "existing statutes provide a basic network of agency jurisdiction over both research and products; this network forms the basis of a coordinated framework and helps assure reasonable safeguards for the public. This framework is expected to evolve in accord with the experiences of the industry and the agencies, and, thus, modifications may need to be made through administrative or legislative actions".

7.3 Legal/Patent Considerations

Patent law is designed to encourage invention by granting inventors a lawful monopoly on their invention normally for a period of seventeen years.

To qualify for patent protection an invention must meet three statutory requirements:

1. It must be capable of being classified as a process, machine, manufacture or composition of matter

2. It must be new, useful and not obvious to someone "skilled in the art"

3. It must be disclosed to the public in sufficient detail to enable a person skilled in the same or the most closely associated area of technology to construct and operate it

For the industries applying the commercial potential of rDNA and hybridoma technology, the interpretation and application of patent law obviously has a profound effect on the way companies run their business. Because of the novelty of the technology,

interpretation of the qualifications for patent protection is complex and one of the major problems emerging within the industry is concerned with patent enforcement.

There are various areas where application for patent could be considered. One could consider patenting an organism, a component of that organism, a process, a product or the specific use of a product. The situation pertaining to the development of new organisms made by gene-splicing and capable of producing drugs or other useful products has been clarified by a recent decision of the US Supreme Court. The essential of the Court decision was that new forms of life can indeed be patented. The problem is the interpretation of the words, new, useful, and not obvious and the decision as to whether the entity being considered for patent actually complies with these requirements.

If patenting a microorganism or a specific hybridoma strain the problem of describing the entity in written detail has been overcome by the depository requirement, whereby samples are placed in a public culture collection and held in confidence until the patent expires. The question of segregation of variants during the holding time does not appear to have been specifically addressed, but any patent granted would be only relevant to the particular strain and is therefore likely to be of limited value.

Patenting a cloned gene or synthetic gene may provide specific advantages because these components are the critical elements of genetic engineering. However, in many cases, it would be difficult to prove that these elements comply with the unobvious requirement.

There is probably a greater opportunity to obtain claims of broad coverage when patenting a process. The weakness of method claims is that they are more difficult to enforce. It may be hard to prove that a potential infringer has actually utilized your method. Frequently it is possible in this type of industry for a competitor to devise alternative methods to achieve the same

functional result. Finally there is always the possibility that a foreign competitor utilizing the method outside the country, may sell the product of the method in this country without liability for infringement.

Patenting a product should, in theory, cause fewer problems. A patent application for a product must provide as detailed a set of functional parameters as one can obtain experimentally, it may then be possible to obtain claims providing broad coverage for variants or equivalents that a competitor may develop.

Claims to method of use lie within the more conventional area of patent practice. Immunoassay techniques and diagnostic kits, for example, have been the subject of many patents and here the new technology does not add new legal issues with respect to such claims.

Opinions vary among spokesmen for the genetic engineering companies on the value of patents in this field. There are statements that such patents are crucial to the development of the industry; other opinions are less enthusiastic, perhaps favoring the use of trade secrecy. Trade secrecy is derived from the common law, as opposed to being specifically created by statute; the state courts recognize and protect it as a form of property. The underlying policy is one of preventing unfair competition or unjust benefits and it is possible that greater protection may often lie in keeping a process secret even though it may qualify for patent. This is especially true for a process that is only a minor improvement in the state of the art or that produces an unpatentable product already made by many competitors.

Patents have been issued in various areas of biotechnology but interpretation of coverage is vague. Defense of the patent rights is the responsibility of the patent holder, not the patent office. Thus, if an infringing party is a large company, litigation can well be prolonged and very expensive. There has already been vigorous and acrimonious legal action concerning

products discussed in this review. Because of the difficulties in interpretation and the strength of the competition between companies it seems almost impossible to ensure patent enforcement in certain areas. It appears, perhaps for these reasons, that some companies are making the decision not to rely on patents but are turning more towards trade secrecy to protect their interests.

8.0 Implications for the Pharmaceutical Industry

Pharmaceutics cannot generally be exercised as a pure science, consequently the pharmaceutical industry has evolved through the integration of various scientific fields. Because of their financial capacity, their marketing and distribution experience, the large pharmaceutical companies have a distinct advantage over many of the small or academic-developed biotechnology companies and many have decided to incorporate biotechnology into their repertories by various strategies (see section 2.3).

Extensive funds have been invested in the field and much more will be needed in order to fulfill the possibilities of applying biotechnology to drug and diagnostics development. In addition resources must be available to prove clinical utility, safety and efficacy, to commercialize the product and hence receive reimbursement. Competitive barriers are forming rapidly in terms of capital, technology, timing, marketing, and distribution. Ability to adapt quickly and decisively to changing business conditions, anticipate market needs, adequately resource critical technologies, and develop new technologies are necessary requisites for achieving competitive advantage. Sufficient resources must be available to attain near term product milestones as well as to ensure success in future programs.

Those medical/pharmaceutical products produced by biotechnology that are currently on the market or in later stages of clinical trials have been mentioned earlier in this review (see

section 2.3). It is possible, however, that these accomplishments will only be the beginning of a new era in therapeutics. Achievements in hand have raised expectations for a large number of diverse products and product concepts that are less well developed and some that are mere speculation. Assessing the future, we must realize that successful applications so far have mainly been based on proteins already identified and studied. Demonstration of clinical efficacy and utility has thus, in many cases, been straightforward. It is extremely unlikely that new entities will be so easily successful.

In order to fully utilize the new developments in biotechnology fundamental scientific research must be done to elucidate specific mechanisms of disease and to improve knowledge of protein structure and function and cellular differentiation. In this way potential therapeutic agents could then be identified or designed by logical sequences.

There is little doubt that rDNA and hybridoma technology could provide a revolution in therapy and diagnosis. The investment climate is favorable but, it appears, only for those companies which have already invested in the early developments, which understand the technical difficulties, are prepared to work closely with the regulatory agencies, and which have the necessary marketing and distribution capabilities.

The prospects for future success are encouraging but we should be equally realistic about the extensive amount of work remaining.

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GLOSSARY

Bacteriophage - A virus that multiplies in bacteria.

Chimera - An individual composed of a mixture of genetically different cells.

Clone - Progeny derived by asexual reproduction from a single individual. Unless there has been mutation or other sporadic change, every individual within a clone is identical.

Endotoxins - Complex molecules (lipopolysaccharides) that compose an integral part of the cell wall, and are released only when the integrity of the cell is disturbed.

Epitope - The small portion of the antigenic molecule specifically recognized by an antibody.

Exotoxins - Proteins produced by bacteria that are able to diffuse out of the cells; generally more potent and specific in their action than endotoxins.

Histocompatibility - Histocompatibility antigens are found on the surface of nucleated cells that provoke allograft rejection and regulate immune responses. Each individual animal possesses its own characteristic set of histocompatibility antigens.

Myeloma - Operationally synonymous with plasmacytoma. Malignant proliferation of B lymphocytes or plasma cells often producing monoclonal populations of immunoglobulins or immunoglobulin fragments.

Oncogene (ONC GENE) - A gene is the genome of some retroviruses that is not necessary for viral replication but is necessary for the ability of the viruses to cause cell transformation in-vitro and rapid induction of tumors in-vivo.

Pathogen - A specific causative agent of disease.

Retrovirus - Small RNA virus consisting of virally coded glycoprotein in a lipid membrane derived from host cell membranes and on RNA nucleoprotein core. The small genome of these viruses contain a gene coding for reverse transcriptase (that directs the production of a DNA copy of RNA), virion proteins and often an oncogene.

REFERENCES

Because of the large number of articles reviewed, references have been divided into sections that as closely as possible parallel those used in the text. General references have been included in section 1.

Section 1

Edmond, S. K., Grady, L. T., Outschoorn, A. S., Rhodes, C. T.,(1986). Monoclonal Antibodies as Drugs and Devices, Drug Develop. Ind. Pharm., 12 (1 and 2), 107-228.

Elander, R. P., (1985). Biotechnology: Present and Future Roles in the Pharmaceutical Industry, Drug Develop. Ind. Pharm., 11:5, 965-999.

"Hormone Drugs," Proceedings of FDA-USP Workshop, May 1982, United States Pharmacopeia Convention, Inc., Maryland, (1982).

"Impacts of Applied Genetics, Microorganisms, Plants and Animals," United States Congress, Office of Technology Assessment, Washington, D. C. (1981).

"Insulins, Growth Hormone and Recombinant DNA Technology," ed., John L. Gueriguian, Raven, New York, (1981).

"Monoclonal Hybridoma Antibodies: Techniques and Applications," ed., John G. R. Hurrell, CRC, Florida, (1982).

"Recombinant DNA Products: Insulin, Interferon and Growth Hormone," ed., Arthur P. Bollon, CRC, Florida, (1984).

Watson, J. D., Tooze, J., Kurtz, D. T., "Recombinant DNA, a Short Course," Scientific American, New York, (1983).

Section 2

Berman, P. W., Lasky, L. A., (1985). Engineering Glycoproteins for use as Pharmaceuticals, Trends Biotech., 3:2, 51-53.

Blundell, T., Sternberg, M. J. E., (1985). Computer-aided Design in Protein Engineering, Trends Biotech., 3:9.

Burke, D. C., (1985). The Interferons, Br. Med. Bulletin, 41:4, 333-338.

Depinho, R. A., Feldman, L. B., Scharff, M. D., (1986). Tailor-Made Monoclonal Antibodies, Ann. Intern. Med., 104, 225-233.

Harris, W. J., Strategies for Selection of Appropriate Biotechnologies for Industrial Applications, in "Biotechnology Applications and Research," Technomic, eds., Cheremisinoff and Ouellette, (1985), 9-25.

Hinman, A. R., Bart, K. J., Orenstein, W. A., (1985). New Vaccines, Int. J. Epid., 14: 4, 502-503.

Hitzeman, R. A., Leung, D. W., Perry, L. J., Kohr, W. J., Levine, H. L., Goeddel, D. V., (1983). Secretion of Human Interferons by Yeast, Science, 219, 620-625.

Inada, Y., Yoshimoto, T., Matsushima, A., Saito, Y., (1986). Engineering Physicochemical and Biological Properties of Proteins by Chemical Modification, Trends Biotech., 4:3, 68-73.

Johnson, I. S., (1983). Human Insulin from Recombinant DNA Technology, Science, 219, 632-637.

Keus, J. A. R., (1986). An Accidental Human Trial of Recombinant Vaccina Virus: A Step Towards Acceptance of Live Recombinant Vaccines?, Trends Biotech., 4:5.

Khachatourians, G. G., Haffie, T. L., Biotechnology: Applications to Genetics, in "Biotechnology Applications and Research," Technomic, eds., Cheremisinoff and Ouellette, (1985), 243-249.

Klausner, A., (1986). Researchers Probe Second-Generation t-PA, Bio/Tech., 4, 706-711.

Nowinski, R. C., Tam, M. R., Goldstein, L. C., Stong, L., Kuo, C., Corey, L., Stamm, W. E., Handsfield, H. H., Knapp, J. S., Holmes, K. K., (1983). Monoclonal Antibodies for Diagnosis of Infectious Diseases in Humans, Science, 219, 637-644.

Sadee, W., (1986). Protein Drugs: A Revolution in Therapy?, Pharm. Res., 3:1, 3-6.

Scott, M. G., (1985). Monoclonal Antibodies-Approaching Adolescence in Diagnostic Immunoassays, Trends Biotech., 3:7, 170-174.

Ulmer, K. M., (1983). Protein Engineering, Science, 219, 666-670.

Van Brunt, J., (1986). Neuropeptides: The Brain's Special Messengers, Bio/tech., 4.

Van Brunt, J., (1986). Protein Architecture: Designing from the Ground Up, Bio/tech., 4, 277-283.

Vournakis, J. N., Elander, R. P., (1983). Genetic Manipulation of Antibiotic-Producing Microorganisms, Science, 219, 703-708.

Yelverton, E., Norton, S., Obijeski, J. E., Goeddel, D. V. (1983). Rabies Virus Glycoprotein Analogs: Biosynthesis in Escherichia coli, Science, 219, 614-619.

Section 3

Arathoon, W. R., Birch, J. R., (1986). Large-Scale Cell Culture in Biotechnology, Science, 232, 1390-1395.

Bebbington, C., Hentschel, C., (1985). The Expression of Recombinant DNA Products in Mammalian Cells, Trends Biotech., 3:12.

Birch, J. R., Boraston, R., Wood, L., (1985). Bulk Production of Monoclonal Antibodies in Fermenters, Trends Biotech., 3:7, 162-166.

Bok, S. H., (1985). Improving the Production of Recombinant DNA Proteins Through Fermentation Development, Pharm. Manufac., Jan., 40-42.

Borrebaeck, C. A. K., (1986). In vitro Immunization for Production of Murine and Human Monoclonal Antibodies: Present Status, Trends Biotech., 4:6, 147-152.

Browne, M. J., Dodd, I., Carey, J. E., Chapman, C. G., Robinson, J. H., (1985). Increased Yield of Human Tissue-Type Plasminogen Activator Obtained by Means of Recombinant DNA Technology, Thromb. Hemost., 54:2, 422-424.

Burnett, K. G., Martinis, J., Bartholomew, R. M., Production of Bifunctional Antibodies by Hybridoma Technology, in "Biotechnology

Applications and Research," Technomic, eds., Cheremisinoff and Ouellette, (1985), 401-409.

Caulcott, C. A., Rhodes, M., (1986). Temperature-Induced Synthesis of Recombinant Proteins, Trends Biotech., 4:6, 142-146.

Chisti, Y., Moo-Young, M., (1986). Disruption of Microbial Cells for Intracellular Products, Enzyme Microb. Technol., 8:4, 194-204.

Cooney, C. L., (1983). Bioreactors: Design and Operation, Science, 219, 728-733.

Crawford, M. A., Johnson, R. W., (1985). Contamination Control in a Class 100 Tissue Culture Media Production Facility, Pharm. Manufac., Jan., 33-37.

Duff, R. G., (1985). Microencapsulation Technology: A Novel Method for Monoclonal Antibody Production, Trends Biotech., 3:7.

Feder, J., Tolbert, W. R., (1983). The Large-Scale Cultivation of Mammalian Cells, Scientific American, 248:1, 36-43.

Fleischaker, R. J., (1986). Applying Large-Scale Culture Techniques to the in vitro Production of Monoclonal Antibodies, Pharm. Manufac., Jan., 16-21.

Giorgio, R. J., Wu, J. J., (1986). Design of Large Scale Containment Facilities for Recombinant DNA Fermentations, Trends Biotech., 4:3, 60-65.

Hartmeier, W., (1985). Immobilized Biocatalysts - From Simple to Complex Systems, Trends Biotech., 3:6, 149-152.

Jarvis, A. P., Grdina, T. A., (1983). Production of Biologicals from Microencapsulated Living Cells, Biotechniques, March-April, 22-27.

Jay, E., Rommens, J., Jay, G., Synthesis of Mammalian Proteins in Bacteria, in "Biotechnology Applications and Research," Technomic, eds., Cheremisinoff and Ouellette, (1985), 388-399.

Karubie, I., Biosensors in Fermentation and Environmental Control, in "Biotechnology Applications and Research," Technomic, eds., Cheremisinoff and Ouellette, (1985), 135-155.

Kronenberg, L. H., Interferons: Manufacture and Application in Medicine, in "Biotechnology Applications and Research," Technomic, eds., Cheremisinoff and Ouellette, (1985), 451-461.

Mizrahi, A., (1986). Biologicals from Animal Cells in culture, Bio/tech., 4, 123-127.

Muth, W. L., Large-Scale Fermentation development of Recombinant Microorganisms, in "Biotechnology Applications and Research," Technomic, eds., Cheremisinoff and Ouellette, (1985), 156-164.

Posillico, E. G., (1986). Microencapsulation Technology for Large-Scale Antibody Production, Bio/tech., 4, 114-117.

Ratafia, M., (1986). Current Issues in the Scale-Up of Biotechnology Processes, Pharm. Tech., June, 42-52.

Reuveny, S., Zheng, Z. B., Eppstein, L., (1986). Evaluation of a Cell Culture Fermenter, Am. Biotech. Lab., Jan/Feb, 28-39.

Van Brunt, J., (1985). Scale-Up: The Next Hurdle, Bio/tech., 3:5, 419-424.

Van Brunt, J., (1986). Fermentation Economics, Bio/tech., 4:5, 395-401.

Van Brunt, J., (1986). Immobilized Mammalian Cells: The Gentle Way to Productivity, Bio/tech., 4:6, 505-510.

Weetall, H. H., Pitcher, W. H., (1986). Scaling Up an Immobilized Enzyme System, Science, 232, 1396-1403.

Sections 4, 5 and 6

Albert, W. H. W., Krueger, D., (1986). Quality Assurance of Therapeutics Based on Monoclonal Antibodies, An European Viewpoint. Boehringer Mannheim GmbH, Biochemical Research Center, D-8132, Tutzing, Germany, F. R.

Alving, B. M., Finlawn, J. S., (1979). Immunoglobulins: Characteristics and Use of Intravenous Preparations, FDA, Bureau of Biologics.

Bethell, D. R., Dawson, M., Lafoe, M. J., (1985). Characterization of Monoclonal Antibodies to Cell Surface Antigens by Particle Concentration Fluorescence Immunoassay (PCFIA), Biotechniques, 3:6, 466-473.

Birch, J. R., Hill, C. R., Kenney, A. C., Affinity Chromatography: Its Role in Industry, in "Biotechnology Applications and Research," Technomic, eds., Cheremisinoff and Ouellette, (1985), 594-605.

Collen, D., Rijken, D. C., Van Damme, J., Billiau, A., (1982). Purification of Human Tissue-Type Plasminogen Activator in Centigram Quantities from Human Melanoma Cell Culture Fluid and its Conditioning for Use in-vivo, Thromb., Hemost., 48:3, 294-296.

Fung, H-L., Chapter seven in "Modern Pharmaceutics," eds., Gilbert S. Banker and Christopher T. Rhodes, Marcel Dekker Inc., (1979).

Gooding, R. P., Bristow, A. F., (1985). Detection of Host-Derived Contaminants in Products of Recombinant DNA Technology in E. coli: A Comparison of Silver Staining and Immunoblotting, J. Pharm. Pharmacol., 37, 781-786.

Grau, U., (1985). Fingerprint Analysis of Insulin and Proinsulins, Diabetes, 34, 1174-1180.

Gubler, U., Chua, A. O., Stern, A. S., Hellmann, C. P., Vitek, M. P., Dechiara, T. M., Benjamin, W. R., Collier, K. J., Dukovich, M., Familletti, P. C., Fiedler-Nagy, C., Jenson, J., Kaffka, K., Kilian, P. L., Stremlo, D., Wittreich, B. H., Woehle, D. Mizel, S. B., Lomedico, P. T., (1986). Recombinant Human Interleukin 1 : Purification and Biological Characterization, J. Immunol., 136:7, 2492-2497.

Hayakawa, T., Kawamura, J., (1983). The Product Quality Assurance Criteria for the Medically Important Polypeptides Produced by Recombinant DNA Technology, Iyakuhin Kenkyu, 14:2, 171-191.

Hou, K. C., Mandaro, R. M., (1986). Bioseparation by Ion Exchange Cartridge Chromatography, BioTechniques, 4:4, 358-367.

Hunkapiller, M. W., Hood, L. E., (1983). Protein Sequence Analysis: Automated Microsequencing, Science, 219, 650-659.

Hustedt, M., Kroner, K. H., Menge, U., Kula, M-R., (1985) Protein Recovery Using Two-Phase Systems, Trends Biotech., 3:6, 139.

Kadam, K. L., (1986). Reverse Micelles as a Bioseparation Tool, Enzyme Microb. Technol., 8, 266-273.

Kirkwood, T. B. L., (1977). Predicting the Stability of Biological Standards and Products, Biometrics, 33, 736-742.

Kurokawa, M., (1979). J. Biological Standardization, 7, 31-41.

"Monoclonal Antibodies: Standardization of their Characterization and Use." Developments in Biological Standardization. Vol. 57 (1985), ed., The International Association of Biological Standardization.

Patel, P. R., Enzyme Isolation and Purification in "Biotechnology Applications and Research," Technomic, eds., Cheremisinoff and Ouellette (1985), 534-562.

Porterfield, R. I., (1984). Application of kinetic models and Arrhenius methods to product stability evaluation, Pharm. Technol., 45-50.

Proceedings of the 26th Annual Conference of Pharmaceutical Analysis, July 1986, Merrimac, Wisconsin: sponsored by The School of Pharmacy, University of Wisconsin-Madison.

Rhodes, C. T., (1984). An overview of kinetics for the evaluation of pharmaceutical systems, Drug Develop. Ind. Pharm., 8 and 9, 1163-1174.

Rhodes, W. E., (1985). Monoclonal Antibodies for in-vivo Use: In-Process and Final Testing, Pharm. Manufac., April, 12-14.

Rhodes, W. E., (1985). Routine Release Testing for in-vivo Monoclonal Antibodies, Pharm. Manufac., May, 12-14.

Rijken, D. C., Collen, D., (1981). Purification and Characterization of the Plasminogen Activator Secreted by Human Melanoma Cells in Culture, J. Biol. Chem., 256: 13, 7035-7041.

Roseto, A., Guillemin, M-C., Chehimi, J., Mazeron, M. C., Peries, J., (1984). Elimination of Mycoplasma, Bacteria, and Fungi Contaminants of Hybridoma Cultures by Intraperitoneal Passage in the Mouse, Hybridoma, 3:3, 297-299.

Sofer, G. K., (1986). Current Applications of Chromatography in Biotechnology, Bio/tech., 4, 712-715.

"Standardization and Control of Biologicals Produced by Recombinant DNA Technology." Developments in Biological Standardization Vol. 59 (1985), ed., The International Association of Biological Standardization.

Sulkowski, E., (1985). Purification of Proteins by IMAC, Trends Biotech., 3:1, 1-6.

Tydeman, M. S., Kirkwood, T. B. L., (1984). Design and analysis of accelerated degradation tests for the stability of biological standards, J. Biological Standardization, 12, 195-206.

Wolfe, A. J., Worthington, H. F. C., (1976). Use of the Arrhenius equation, Drug Develop. Ind. Pharm., 1, 185-194.

Wong, B. L., Charm, S. E., (1983). Product Recovery from Genetically Engineered Organisms: A Review of the Practical Problems, Genet. Eng. News, March/April.

Section 7

Benson, R. H., (1986). Biotechnology Patent Pit Falls, Bio/tech., 4.

Bliem, R., (1986). ESACT '85 Meeting Report, Products from transformed Cell Lines: The Stance of WHO and FDA, Trends Biotech., 4:1, 1-2.

Gartland, W. J., Government Guidelines in "Biotechnology Applications and Research," Technomic, eds., Cheremisinoff and Ouellette (1985), 29-40.

Gibbs, J. N., (1986). Regulating Bioengineered Veterinary Drugs, Bio/tech., 4, 414-416.

Halperin, J. A., (1985). Helping Drug Regulatory Authorities to Help You, Clin. Res. Practices and Drug Reg. Affairs, 3:2, 167-184.

Korwek, E. L., (1982). FDA, OSHA, and EPA Regulation of the Recombinant DNA Technology, J. Parenteral Sci. Technol., 36:6, 251-255.

Linhardt, R. J., (1986). Protein Engineering and Site-Directed Mutagenesis, Patents, Applied Biochem. Biotech. 13, 75-79.

Miller, H. I., (1982). Recombinant DNA as a Paradigm of New Technology: Its Impact on Regulation by the Food and Drug Administration, J. Parenteral Sci. Technol., 36:6, 248-250.

Rhodes, W. E., (1985). Monoclonal Antibodies as Drugs: A Manufacturing and Regulatory Overview, Pharm. Manufac., Jan

Petricciani, J. C., (1985). Regulatory Considerations for Products Derived from the New Biotechnology, Pharm. Manufac., March, 31-34.

Federal Register, Vol. 51, No. 123, Thursday, June 26, 1986. Part II, Office of Science and technology Policy, "Coordinated Framework for Regulation of Biotechnology: Announcement of Policy and Notice for Public Comment."

Points to Consider in the Production and Testing of New Drugs and Biologicals Produced by Recombinant DNA Technology. (1985). Office of Biologics Research and Review, Center for Drugs and Biologics, Food and Drug Administration (FDA), Bethesda, MD 20205.

Points to Consider in the Manufacture of Monoclonal Antibody Products for Human Use (1983). Office of Biologics, FDA.

Points to Consider in the Manufacture of in-vitro Monoclonal Antibody Products Subject to Licensure. (1983). Office of Biologics, FDA.

Points to Consider in the Production and Testing of Interferon Intended for Investigational Use in Humans (1983). Office of Biologics, FDA.

Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals (1984). Office of Biologics, FDA.

Guideline on General Principles of Process Validation (1984). Division of Drug Quality Compliance, Office of Drugs, FDA.

Section 8

Blumenthal, D., Cluck, M., Louis, K. S., Stoto, M. A., Wise, D., (1986). University-Industry Research Relationships in Biotechnology: Implications for the University, Science, 232, 1361-1366.

Dibner, M. D., (1986). Biotechnology in Europe, Science, 232, 1367-1372.

Dibner, M. D., (1985). The Biotech Connection, Pharm. Exec. Sept., 81-84.

Elander, R. P., (1985). Biotechnology: Present and Future Roles in the Pharmaceutical Industry, Drug Develop. Ind. Pharm., 11:5, 965-999.

Evans, S., Grassam, P., (1986). Considerations for Parenteral Dosage Form Development of Natural Alpha Interferon, J. Parenteral Sci. Technol., 40:3, 83-87.

Harford, S., (1983). Recombinant DNA Technology and The Pharmaceutical Industry, The Pharm. J., London, Sept. 17, 320.

Klausner, A., (1985). Words of Caution as Monoclonals Mature, Bio/tech., 4, 604.

Murray, J. R., (1986). The First $4 Billion is the Hardest, Bio/tech., 4, 293-296.

Norris, C. E., (1986). Tax and the Business of Biotechnology, Trends Biotech., 4:7, 169-172.

Prescott, L., (1983). Hybritech: Portrait of a Monoclonal Specialist, Bio/tech., April, 156-161.

Van Brunt, J., (1986). Contract Production: Buying Technical Expertise, Bio/tech., 4, 701-705.

Proceedings of the Educational Seminar, "The Impact of Hybridoma Technology on the Medical Device and Diagnostic Product Industry," Arlington, Virginia, June 1982. Health Industry Manufacturers Association, HIMA Report No. 82-1.

Biotechnology Newsletters

Applied Genetics News
Biomass Bulletin
Biomass Digest
Biotech News
Biotech Quarterly
Biotech Update
Biotechnology Bulletin
Biotechnology Bulletin Reports
Biotechnology Information
Biotechnology Investment Opportunities
Biotechnology Law Report
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Biotechnology Patent Digest
Biotechnology Press Digest
Genetic Engineering and Biotechnology Monitor
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