General Introduction

Developments in recombinant DNA technology over the last decade have made possible the production of many scarce human proteins in quantities unavailable before. It has also become clear in the past five years that mammalian cells can effect many post-translational modifications (e.g.,Glycosylation, fatty acylation, sialylation, β-hydroxylation and γ-carboxylation etc. , to name a few) that are normally found in human proteins. While some may appear to be dispensable for biological activity *in vitro* , they may nevertheless influence the physiological and antigenic properties of the protein. There are also several instances where these modifications are essential for bio-activity. Accurate polypeptide folding, efficient assembly of sub units, and secretion of the mature form of these proteins are the additinal reasons why it is desirable in some instances, and obligatory in others, to use engineered mammalian cells for protein production ; especially so when the product is destined for human use as a therapeutic.(Ramabhadran,T.V.'1987).

The use of human or animal derived products as therapeutics has experienced a long and productive history. Vaccines, one of the first such products to be used for human and veterinary application, were prepared originally in whole animals but the techniques to grow single animal cells in culture and the demonstration that these cells can support virus replication ushered in the era of vaccine production from cell cultures(Spier ,R.E. and Griffiths, J.B.'1985).Since 1954, when the Salk vaccine for polio-virus was first to be produced by cell culture, human vaccines against mumps,rabies , measles and several other veterinary vaccines have been derived from cell cultures.Cells in culture also produce enzymes ,hormone and growth factors but only cell-line derived from tumours can usually produce these poly-peptides indefinitely. Most primary cultures established from normal tissue have a limited life span *in vitro* and lose the ability to produce the protein of interest(Eagle,H.'1965).Even in most of the tumour cell-line derived therapeutic poly-peptides, the production level is too low for commercial exploitation (Griffiths,J.B.'1985).

However, two recent advances in biotechnology ie..Monoclonal antibody production by hybridoma technology and other protein production by recombinant DNA technology , have given new impetus to exploitation of mammalian cells for producing human polypeptides as biological drugs. Mammalian cells being the natural sources of many therapeutic proteins have rendered possible high-level therapeutic protein production from genetically engineered mammalian cells. In conjuction with the technologies of high-density cell-culture systems (e.g. Perfusion Microcarrier Bioreactors) the cost effectiveness of the product has also been made comparable to those derived from other recombinant systems(Kaufmann,R.J., et al'1987.)

In the world scenario,the developments in biotechnology and the related economic growth has been restricted mainly to developed countries which had adopted conducive policies and programmes for development of new technologies and also made substantial R&D funding from venture capital based industries and goverment agencies together.. Such growth of new biotechnology companies in USA (e.g. Genentech ,Biogen, Chiron and Genetics Inc.) have radically transformed the health care products (pharmaceuticals and diagnostics) industry, only because of the rDNA technology derived products.According to a forecast, the annual market is expected to grow world-wide to $25-30 billion by 2000 A.D.

Table1.1:Estimated market of medical products through biotechnology

(in million $)

Market	Year		
Medical products	**1985**	**1990**	**2000**
Pharmaceuticals	-	3500	20000-30000
Diagnostics	100	1500	5000
Veterinary products	100	1500	5000
Others(Sensors, bio-materials etc.)	-	75	500

Source:SRI International.,California,USA.

Till date about 20 drugs have been approved by FDA and some of them have gained outstanding therapeutic prominence. About 100 drugs are in the human trials beside some 400 products in various stages of development. The first human proteins made available through rDNA technology were insulin and human growth hormone, the peptides whose medical and physiological role had been well described.Soon to follow pursuit were the proteins which had hitherto been difficult to be isolated and characterised using conventional means: the interferons,interleukins,colony-stimulating factors,tissue plasminogen activator, to name but a few(Drews,J.'1993). Some of the proteins in development as novel drugs of future have no precedence in nature because they are combinations of certain domains from several proteins. One molecule , for instance , represents the greater part of interleukin-2 fused to those parts of diptheria toxin that are necessary for the translocation of the toxin through endocytic vesicles and for the adenosyl ribosylation of elongation factor-2 (causing inhibition of protein synthesis). Other artificial constructs comprise soluble receptors for cytokines such as TNFs,IL-1, or IL-5 fused to a heavy chain of human IgG. These constructs are expected to bind cytokines produced in large amounts during some disease processes, thus mitigating the resulting pathology.

Table1.2: Recombinant products in clinical development.

Protein	**Number**	**Indications**
Growth Factors (eg.TNF,CSF, EPO, EGF,FGF,PDGF etc.)	27	Cancer,Anaemia,wound-healing, viral and bacterial infections, bone marrow transplantation
Hormones (e.g., Insulin, IGF , hGH, GRF, Relaxin etc.)	13	Diabetes,growth disorders, Osteoporosis.
Interferons	11	Cancer, Viral infections.
Interleukins	19	Cancer., Immunomodulation
Thrombolytics (e.g., tPA)	14	Cardio-vascular diseases
Vaccines	28	Hepatitis-B,AIDS,Malaria, Pertussis, typhus, influenza.
Chimaeric Recombinant proteins	22	multiple
recombinant live vaccine	6	Immunity to specific infections
recombinant monoclonal Abs.	11	Cancer, infection, inflammation
Soluble receptors(e.g.,CD-4, IL-1 rec TNF-α receptor)	3	Inflammation, HIV-infection

Source: Pharmaprojects Database, Feb'93.

During the next five years , atleast 10-15 % of revenues and profits derived from new drugs will stem from recombinant proteins and it is imminent to expect a fair increase beyond the five year period. The port folio of recombinant proteins now in clinical trials should amount to $10-20 billion in todays currency, In ten years from now, the total pharmaceutical market is expected to reach $250 billion .So recombinant proteins expected to account for 10-15% of the estimates , will have a market of more than $25 billion atleast.

Table1. 3:Biopharmaceutical World Market(Million $)

Specialities	1995	2000
Cancer	730	1900
AIDS	100	2500
Cardiovascular disease	680	1500
Monoclonal therapeutics	200	490
Growth factors	670	1500

For the next 10 to15 years there will be an abundance of monoclonal antibodies, Ab-toxin conjugates or constructs containing cytokines , cytokine-receptors and antibody heavy chains or other protein components with immuno-suppressive and anti-inflammatory properties. These molecule should carry significant advances in the treatment of rejection episodes after organ-tranplantation,graft versus host disease , acute flare-ups in auto-immune diseases and septic shock. Sometime around 2003 we will witness the arrival of cytokines , and the combined use of cytokines which will improve the prospects of treating certain tumours like renal cell cancers, melanomas, lymphoproliferative diseases and leukemias.The cocomitant use of alpha-interferons and retinoids in treating epithelial cancers represents a typical examples of cytokine in conjunction with chemotherapy while the combination of IL-3 with G-CSF is the other treatment regimen for general immunomodulation following cancer therapy.

Objectives of the present study:

Eukaryotic expression technology has enabled us to express human proteins of therapeutic interest in the heterologous organism. Though, several expression systems have been developed using bacterial cells, lower eukaryotes (eg. yeast), invertebrate insect cells etc. as hosts; mammalian cells probably represent the ultimate choice to mimick the defined post-translational processing of the 'native protein'. This is particularly important where the recombinant product is destined for therapeutic use either through systemic delivery to target site or by somatic cell gene therapy. For development of industrial recombinant cell-lines the present mammalian expression systems need to be refined for their transcriptional /translational efficiency so as to make the product commercialisation economical. These have propelled us to take up research in the field of maximization of expression of therapeutic protein genes cloned in mammalian cells so as to establish a stable high-level mammalian expression system for production of therapeutic biologicals.

It has been envisaged in the project to try for constitutive episomal expression of the cloned genes in conjunction with cooperative enhancement of expression level through interaction of multiple regulatory sequences such as 'cis'and 'trans' acting activators, proper splice signals, translational activators, or the removal of mRNA instability sequences at downstream of the cloned genes. It is also necessary that the host cell environment interacts with such control sequences to express the recombinant protein with desired post-translational modifications.Hence,

choice of host cell-line has been given a special thought with regards to mimicking the natural host cell machinery for the recombinant therapeutic expression ie. a primate cell-line which has been amenable to industrial scale-up for biologics production (e.g. VERO-76).

The reporter gene for the study , is a polypeptide of 17.3 Kd monomer mol.wt. cytokine called "Tumour necrosis factor-α (TNF-α), also known as 'Cachetin' produced by monocytes and macrophages and functions as a multi-potent modulator of immune system activation. The production of TNF-α is mediated by the action of the lymphokines and endotoxins on monocyte/macrophage mostly and the secretion is enhanced by IFN-γ . TNF-α then induces or enhances the specific production of Class I MHC antigens, GM-CSF and IL-1.

The therapeutic potential of TNF-α has been described by Klausner,A et al '1987 in reference to the treatment of certain melanomas, lymphomas, and in breast and Kidney cancers. TNF-α as Therapeutic , is currently in clinical trials(Bollon,A.P. et al'1988) in combination with other cytokines. The levels of TNF-α in this conditions and other conditions may prove to be important in the characterization and monitoring of diseases. Scuderi,P. et al'1986 suggested that increased levels of TNF-α may play a role in the host defense mechanism against parasitic infections. Mestan ,J. et al' 1986 suggested that TNF-α plays a protective role in Viral infections. It has also been shown to be markedly elevated in renal allograft rejections (Maury ,P.J. and Teppo, A.M.' 1987, Mclaughlin, P.J. et al' 1991). Beutler,B. and Cerami,C.'1986 showed that TNF-α is the primary mediator of endotoxin-induced injury to the tissues and septic-shock . It has a significant role in the pathogenesis of inflammatory disease of joints called 'Rheumatoid arthritis' and other auto-immune disorders of tissues.

We have concentrated our efforts to the production of this therapeutic immuno-modulatory cytokine with anti-tumour potential, as our reporter gene for evaluation of high-level expression system to be developed through this project. The reporter constructs have been included through RT-PCR cDNA cloning approach (Belyavsky,A.,et al'1989) and the same has been expressed in a prokaryotic as well as mammalian cell as host systems.

The studies carried-out to achieve the above-mentioned objectives are as follows:

- Ascertain a proper donor human cell-line for mRNAs representing most of the cytokine transcripts.
- Establish a human cytokine cDNA library through a rapid and efficient approach.
- Screen the library for representation of a anti-tumour cytokine ie. TNF-α.
- Check the physical and biological validity of the TNF-α cDNA clones through molecular and prokaryotic expression studies.
- Construct mammalian episomal expression vectors for high level expression of hTNF α, as a representative cytokine reporter gene from the above-mentioned library.
- Transfer the mammalian expression constructs into a proper host cell-line for authentic high-level constitutive expression of hTNF-α.
- Study some characteristic features of the expressed hTNF-α from the recombinant cell-lines developed through this project.
- Ascertain expression levels from the primary clones of recombinant cell-lines developed.

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