# History, Current Status and Future of Recombinant Protein Products

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# Outline - rDNA protein products

- Briefly review the development of rDNA medicinal products
- Regulatory oversight
- Discuss safety and other issues for industry and regulators using real examples and lessons learnt
- Impact of bioimilars
- Future developments
- **This is a personal view**

# Biotechnology Products including rDNA-derived proteins

- Last 30 years seen revolution in DNA-based and related technologies
- Opened new exciting vistas for global public health - disease diagnosis / treatment / prevention / correction defective genes
- Cutting - edge of biomedical research
- Economically fastest growing sector in pharmaceuticals

# History – Traditional Protein Products

- Protein products **isolated** from biological materials - animal, human, microorganisms
- Include vaccines and biotherapeutic proteins
- Hormones and blood products –

  *Animal derived insulin (available since 1920s)*

  *Human pituitary derived growth hormone (late 1950s)*

  *Plasma derived clotting factors eg Factor VIII, (1960s)*
- Limited supplies
- Problems included development of inhibitors of biotherapeutics (antibodies), Viral contamination of Factor VIII (HIV, Hepatitis C virus) and Creuzfeldt Jacob Disease (CJD) of pituitary derived growth hormone

# Quantum Jump

- Sequencing nucleic acids
- Ability to "word process" genes - **"cut, copy, paste" DNA sequences**
- **Express human or microbial genes in foreign cells (bacterial, mammalian, plant, yeast, insect) and produce clinically useful biological macromolecules such as biotherapeutics or antigens**
- **Great progress also been made in ability to purify and to characterize biological macromolecules in great detail**

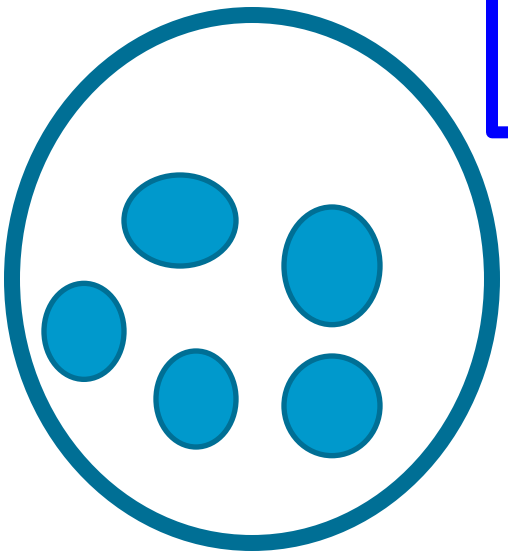
# rDNA-Derived Proteins

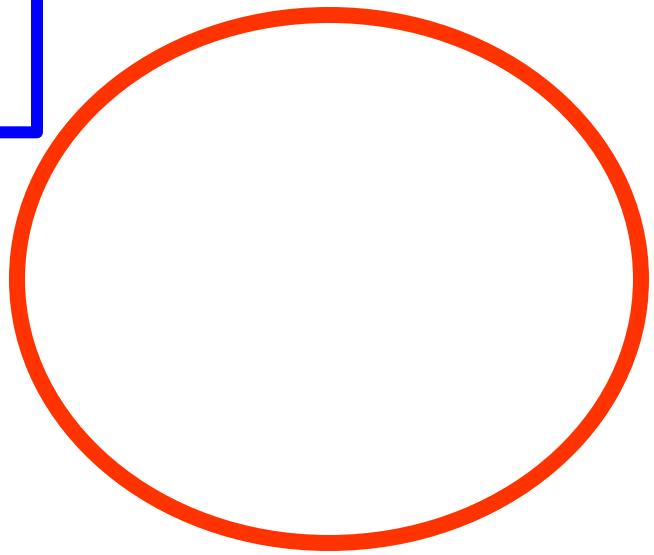
- First came to market in the early 1980s and revolutionized the field of human biotherapeutics, *in vivo* diagnostics and vaccines (eg yeast derived hepatitis B vaccine)
- Originally,the biotherapeutics were **copies of human proteins**, such as insulin or growth hormone
- Then, came **intentionally modified versions** (altering the genetic sequence) with the **view to achieving manufacturing efficiencies or to improve therapeutic outcomes**
- Also rDNA - derived **proteins that do not exist in nature**, such as Monoclonal antibodies and fusion proteins.

# Alterations to DNA sequences Manufacturing efficiencies

- Expression vector built with relevant prokaryotic and eukaryotic controlling elements
- For expression in bacteria, ribosome-binding site and optimally spaced initiation AUG codon recognized by the initiator N formyl-methionine tRNA added to the DNA sequence to be expressed.
- Sequences enabling cleavage of the terminal F Met and methionine also incorporated **(concern about immunogenicity)**
- Signal sequences for exporting product out of the cell for easier processing can be added
- Deletions / insertions in wild type DNA to increase protein yield ( eg deletion B domain Factor VIII)
- Optimizing codon usage to facilitate translation - yield

Constitutive or induced expression





rDNA protein as inclusion bodies inside cells. Isolated from harvested cells, unfolded and refolded as active protein - Downstream processing and purification

rDNA derived protein secreted. Cells removed and supernatant used for isolation of rDNA derived protein

Downstream processing – isolation and purification

# Alterations to DNA sequences and other changes to improve therapeutic outcomes

- Fusion proteins – biological activity of one protein combined with another eg extending serum half life by attaching an immunoglobulin Fc domain
- Covalent attachment of polyethylene glycol (PEG) can also be used for increasing half life of a product (controlled Pegylation only at specific sites following site directed mutagenesis)
- Site directed changes can increase number and location of glycosylation sites leading to increased negative charge and enhanced hydrodynamic size reducing renal clearance – eg engineered erythropoetin
- Humanizing amino acid sequences to minimize immunogenicity eg single domain Ab from camelids

# The Future

- Novel rDNA proteins - fusion proteins directed at specific targets, humanized single domain antibodies (nanobodies).
- New therapeutic proteins for unmet needs - cancer, Alzheimer Disease
- For biotherapeutics, immunogenicity still likely to be a major safety issue but whether inhibitory uncertain.
- Longer term may see  traditional rDNA proteins being replaced by gene therapy – correcting faulty genes
- New vaccine antigens and monoclonal antibodies especially against pathogens which might cause public health emergencies

# Regulatory oversight

- **REGULATORY MEASURES** put in place **very early on** in development of biotechnology products - **regulated as biologicals** (eg Canada, Europe, USA)
- **GUIDELINES** on production and quality control rDNA derived proteins **also developed early on** (eg EMA, FDA, WHO)
- **Based on experience with biologicals** in general; provided framework for moving forward with newer biotechnologies and products
- Original guidelines have been updated over time and developed by many other agencies

# What are the issues?

- **Differ from Chemical Drugs in many ways**
- Biological starting materials and /or manufacturing process - **inherently variable**
- Highly complex products eg large protein molecules, often glycosylated : cannot be **fully** characterized by physicochemical tests **alone**
- Rely on biological test methods (bioassays) to characterize product - potency (activity), immunogenicity, safety ; **inherently variable**
- **Standardization** of processes essential. **International physical standards / reference reagents** play an important part in controlling the performance of assays for bioactivity evaluations

# Critical Manufacturing Points

- Expression system – mammalian cells, bacteria, yeast, insect or plant cells etc
- Cell culture / fermentation / genetic stability : batch or continuous production
- DNA Sequence / translational events
- Separation and purification of product
- Characterization of resulting protein + glycosylation or other modifications
- Bulk product testing (drug substance)
- Formulation
- Final product testing (drug product)
- **Slight changes in process can have major effects on clinical performance of the product. Consistency of production critical**

# Product characterization

- Means **more** than simple quality control tests
- Expect **several** parameters to be evaluated by different techniques, **not just one**
- Protein sequence, secondary / tertiary aspects, glycosylation, phosphorylation, oxidation, lipidation, etc
- Product / **host cell** related impurities (including **residual DNA from continuous cell lines**; **Viral safety validation)**
- Potency ( biological activity)
- Formulation implications and Stability

# Non-clinical and Clinical Evaluation

- **Non clinical**
- Testing in animals of limited relevance
- DNA-derived biotherapeutics have unique and diverse structural and biological properties, **including species specificity**, immunogenicity and unpredicted pleiotropic activities.
- Pharmacological and safety evaluation need to take a large number of factors into account . Flexibility needed - case by case approach
- **Clinical**
- Extensive evaluation covering safety and efficacy, including  immunogenicity studies

# Factors affecting expression of a foreign gene in a new host cell

- Copy number of gene
- Strength of promoters
- Controls on gene expression - induced or constitutive
- Choice of codons in cloned gene (original or modified to match cell type)
- Genetic stability of recombinant system

# GENETIC STABILITY ISSUES

## Examples and lessons learnt

# a) Beware of the "we know it all attitude"

- rDNA derived insulin submitted for licensing in **UK** early 1980s
- Quality controls proposed by manufacturer lacked suitable in-process controls
- RELUCTANTLY manufacturer agreed to include end of fermentation assay for plasmid characterization – interesting outcome

**(gene for insulin on a plasmid in *E coli*)**

# Expect the Unexpected

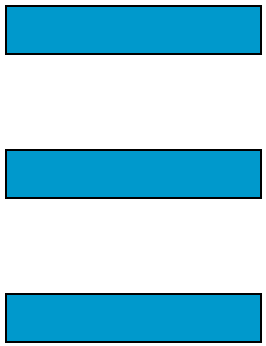
- New quality control step involved examining plasmid by sizing
- Data sent to NIBSC for review
- Over 400 very large scale fermentations carried out successfully over 12- 18 months
- UK regulator about to relax control and declare system stable ( only manufacturer to review data)
- **Surprise**

# Expect the Unexpected

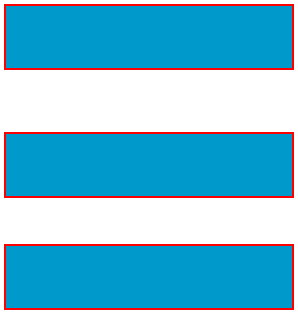
- Surprise very poor product yield ( 20 – 30% normal) in one large fermentation
- All other parameters normal, bacterial growth, fermentation characteristics (temperature, oxygenation)
- The newly introduced quality control measure enabled rapid resolution of the problem

# Plasmid sizing on electrophoresis gel

## Separating DNAs and checking their size



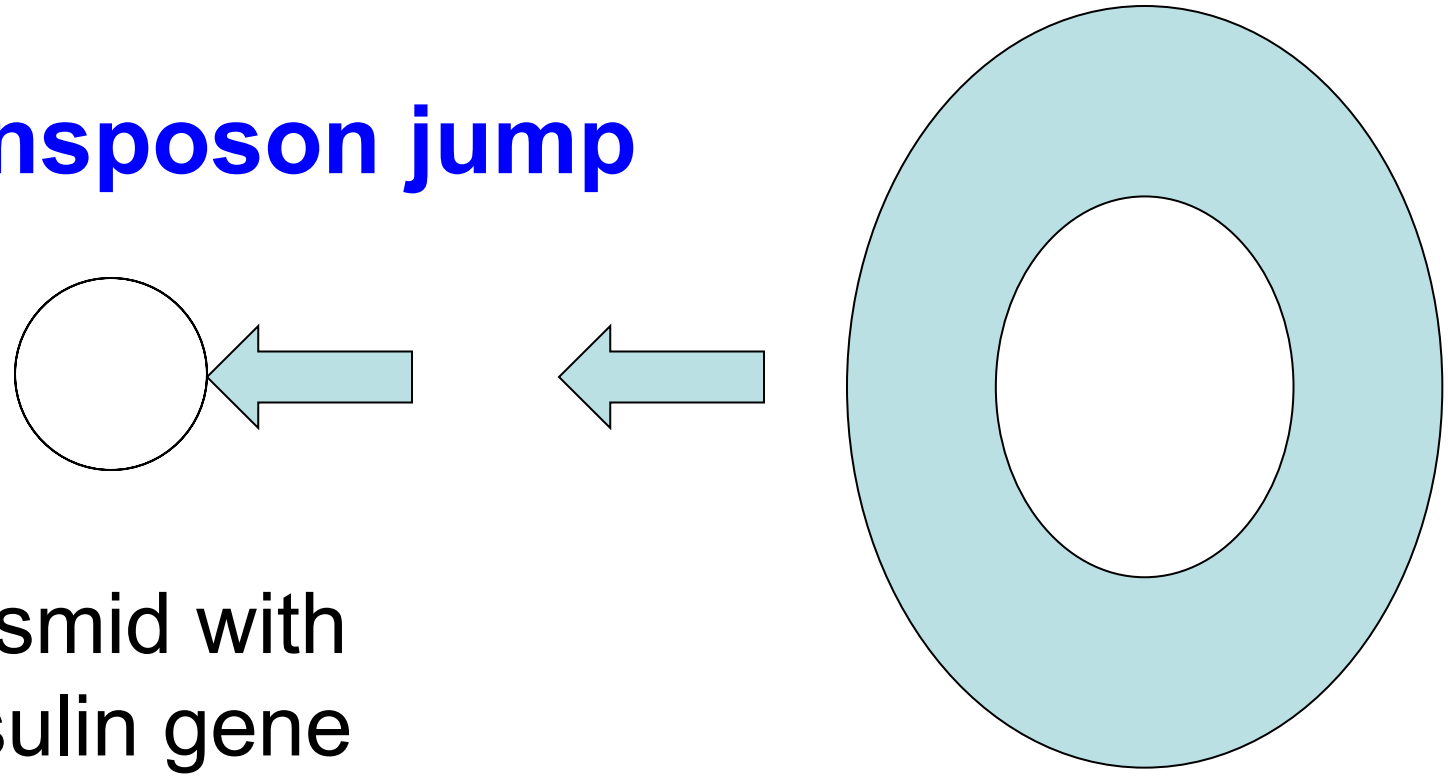
routine



**unexpected**

# •Jumping Genes

**transposon jump**



plasmid with
insulin gene

chromosome

# Expect the Unexpected

- Transposon inserted into operator of the insulin gene shutting down expression in majority of the organisms
- System had been biologically stable for long time – during product development and 12-18 months of production experience

# Outcomes

- Led to manufacturers screening production strains of *E coli* for transposable elements and deleting them
- Problem eliminated by major manufacturers since only transposon free strains now used for development and production of rDNA products
- **Pay attention to starting materials**

# b) Recombinant gene sequence and Product Consistency

- Concern in early 1990s that low level variant DNA sequences in the production gene would lead to variant proteins in the product which might go unnoticed
- Insensitivity of protein characterization
- Worry that the variant proteins might be detrimental by blocking receptors or have unwanted biological properties (eg increased or decreased activity, more immunogenic)

# Recombinant gene sequence and Product Consistency

- Transfected gene sequence itself gave only consensus DNA sequence
- Low level variants might be missed
- Proposal made to **clone the transfected gene many times and sequence each individual clone** to arrive at a statistically sound estimate of variant sequences
- If variant 10%, necessary to sequence 28 clones  to detect with 95% confidence

# Recombinant gene sequence and Product Consistency became an issue

Value of multiple DNA sequencing **questioned** and discussed at number of international meetings –

- Considered it does **not guarantee absence of variant proteins** because of –
- *Possible mutational events during fermentation*
- *Transcriptional errors*
- ***Translational errors***
- *Protein processing effects*

# Translational errors during rDNA protein synthesis

- Known to occur at low level eg in prokaryotic systems like *E coli*
- Expressing foreign proteins at **high rates** to make them major cell components can lead to increased frequency of error rates in production cells - **nutritional stress**
- Error rates in *E coli* about 0.1%. It is 25 times greater during high level synthesis of mouse epidermal growth factor

# Translational errors during rDNA protein synthesis

- **High level synthesis** of Somatropin or interleukin 2 in *E coli* gives rise to errors
- Both have high levels of leucine and over-expression creates unusually high demand for leucine
- *E coli* responds by increasing biosynthesis of leucine which also leads to accumulation of Norleucine
- Norleucine is structural analogue of methionine and gets incorporated into the protein instead of methionine
- **Resolved** by supplying high levels of leucine during fermentation

# Outcomes

- Problems of translational and other errors can be detected only at **the protein product level**
- Emphasis should be placed on detailed product characterization, including characterization of product related variants and their quantity
- Regulatory move to sequence multiple clones of transfected genes lessened
- **Focus on improving technology for protein purification and characterization . Considerable advances since that time.**
- Problem can still occur eg when measures to increase yield introduced, such as induced expression or extension of fermentation time.

# Codon Optimization – unwanted consequences

- Optimization done to improve translational efficiency – increased expression and product yield
- Codon usage also regulates speed of translation and correct protein folding .
- Caution - too rapid translation may lead to incorrect folding and functionality of the resulting protein molecule with potentially safety issues
- **Pay attention to the whole process of rate of protein expression especially if changes are introduced during product development – could impact product going into phase I, II , III clinical studies**

# VIRAL CONTAMINATION

# Viral safety of rDNA products – critical issue

- Many are produced in mammalian cells
- Measures needed to ensure absence of infectious agents in product
- A contaminating virus MIGHT spread from recipient to contacts / community
- Could become serious threat to health of a country or globally – no borders for bugs
- Contamination of cell lines and products also has **huge economic consequences** for manufacturer
- Might lead to **supply issues** with considerable public health impact

# Detailed Guidelines available
# A belts and braces approach

- *WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological products and for the characterization of cell banks (2010)* : ICH, national guidance
- Master cell bank, working cell bank exhaustively screened for virus contamination, with documented history
- Control of raw material used in production – eg growth media
- Closed systems for growth of cell culture
- Testing of each cell culture lot for viruses
- Validation of viral inactivation /removal by downstream processes

# Does the system work?

- Generally yes
- However, viral contamination has occasionally occurred but contained and usually prevented from getting into product on the market
- Seems that not all contaminations are reported publically; manufacturers concerned about bad publicity in media (see Nature 472, (2011) 389-390)
- Some manufacturers have reported contaminations - MVM, Genentech 1993, 1994: Vesivirus 2117 , Boehringer-Ingelheim 2003: Vesivirus 2117, Genzyme (Belgium and USA), 2008: PCV 1 & 2, GSK and Merck, 2010.

# Genentech experience with contamination in cell culture 1993

- Contamination of large scale cell culture by Minute Virus of Mouse (MVM) detected during routine production control process
- Testing takes time and product already well on way through downstream purification processes by time detected
- Lot production stopped, reported to US FDA and clean up started.
- Investigation of source instigated

# Genentech experience with contamination in cell culture 1993

- No definitive source of contamination identified; consistent with media used in production as source. Feral mice from land surrounding plant examined but no MVM found.
- Clean up process expensive
- New PCR and infectivity assay developed to speed up early testing and introduced routinely

# Genentech experience with contamination in cell culture 1994

- New PCR and infectivity assays used and nothing found for 12 months
- Then another positive signal but this time contamination detected before any downstream processing started. **Downstream protected.**
- New testing dramatically minimized impact on downstream processes
- At no time was a contaminated product let through the system and the regulator was aware of all developments

# Genentech experience with contamination in cell culture 1994

- Second MVM found to be sufficiently different in DNA sequence to rule out plant recontamination with residual 1993 virus
- Source highly likely to be contaminated cell culture media but not shown directly
- New heat treatment of medium developed , approved by FDA and installed
- **No viral contamination detected since 1994**

# The Genzyme  Experience

- Detected virus contamination : several bioreactor runs (Belgium and USA, 2008 -2009) terminated due to suspected contamination. Seems not to have dealt with problem promptly.
- US FDA warning letter and re-inspection
- Virus identified as Vesivirus 2117 using PCR in 2009 : not known to be a human health risk but interferes with growth of CHO cells .
- Likely introduced by contaminated media
- USA plant shut down for major clean up and re-organization. Virus had spread into manufacturing facility – bioreactors and expensive chromatography columns. Clean up very costly.

# The Genzyme  Experience

- Global supply of two rDNA derived orphan drugs, Cerezyme ( Gaucher's disease) and Fabrazyme (Fabry's disease), were seriously compromised and the products rationed. No alternative to Fabryzyme.
- Overall Genzyme needed lot of GMP actions, stock prices dived and together with sales shortfall left the company vulnerable to takeover - acquired by Sanofi in 2011

# Outcomes, viral contamination

- **Continued vigilance essential**
- Virus contamination is a serious business.
- Manufacturers need to deal with contamination **promptly** (compare Genentech & Genzyme)
- Alarm bells now ringing across industry. Biotherapeutics was once the domain of speciality biotechnology companies but now products being produced in very large quantities by many manufacturers. Scale up may be more difficult to handle
- Next generation sequencing (deep sequencing) for adventitious virus detection now under consideration.

# Impact of Biosimilars

- Wide agreement that well-established regulatory pathway for authorization of **generic versions** of small molecule drugs **not appropriate for copies of rDNA proteins -** not be identical but similar

- Guidelines from EMA, WHO , Korea, USA , Canada and other agencies available. Some revised 2014-2016

- **Similar Biotherapeutic Product** (SBP) is “similar” in terms of quality, safety and efficacy to an **already licensed Reference Biological Product** (RBP) : similarity based on a **“Totality of evidence”**

# WHO Guidelines (2009)- basic principles

- **Demonstration of similarity** of SBP to RBP **in terms of Quality** is a **pre-requisite** for the reduction of the non-clinical and clinical data set required for licensure
- **Emphasize Head to Head** Comparability Exercise - applies to Quality, Non clinical and Clinical aspects.
- Discuss statistical design and analysis of equivalence / non-inferiority clinical trials for SBPs. Both maybe acceptable
- **Clinical studies** designed to detect possible differences in safety and efficacy between SBP and RBP, not to repeat phase III studies **(confirmatory studies to resolve uncertainties related to biosimilarity)**
- Comparability of the **immunogenicity** of the new and reference product is essential
- Need to justify **extrapolation to other indications,**

# Biosimilars- Extrapolation of Indications

- A Reference Biological Product may have more than one therapeutic indication but the abridged clinical studies **will have studied only one.**
- When biosimilarity has been demonstrated in one therapeutic indication , extrapolation to other indications of the Reference Biological Product **may be possible** – **but not automatic**
- **Must be justified** - mechanism of action, pathophysiological mechanisms of the disease(s) involved, safety profile , level of differences in quality
- Case by case considerations. **Difficult decision with large complex multifunctional products like biosimilar Mabs**

# Biotherapeutics / Biosimilars
# Technological advances

- Technologies now available to characterize complex biological macromolecules in exquisite detail – 3D structure of protein and glycan structures, oxidations and other post translational modifications
- Need to show similarity but also identify differences. There **will be** differences between SBP and RBP – they are *highly similar* but not *identical*.
- Introducing manufacturing changes to approved biotherapeutic products may likewise result in analytical differences between the pre- and post change products
- **Predicting the impact of structural differences, and the level of differences, on biological activity and clinical performance is the difficult part , especially in the case of multifunctional products**

# Challenges - differences between rDNA derived proteins

- **Biosimilars** – we need to be assured that they behave like the innovator molecule in all approved clinical indications
- Quality and biological differences may affect decisions on biosimilarity and on extrapolation of indications of highly complex multifunctional products (Mabs) – how similar is similar?
- **Manufacturing changes** - we need to be assured of the continued quality, safety and efficacy (clinical performance) of the post-change product
- **Better understanding** of the **impact of structural differences** in biological macromolecules on their biological and clinical activities will greatly support comparability evaluation

# Building up a knowledge base

- **Primary structure** . Changes in protein sequence can occur through mistranslations due to manufacturing changes even if the gene sequences of the SBP and RBP, or pre and post change products, are identical. This may alter the levels of variants which will need investigation
- **Charge variants** can affect some but not all products eg oxidation of interferon beta-1a can induce highly immunogenic aggregates
- **Glycosylation** Differences in glycosylation can influence biological activities such as antibody-dependent cellular cytotoxicity (ADCC) (eg level of afucosylation in Mabs), immunogenicity and half life . Glycoforms important.

# Building up a knowledge base

- Much work now ongoing in this area driven by biosimilars issues
- **Moving towards a better understanding the structure / function relationships of rDNA derived proteins**
- Knowing which parameters affect Critical Quality Attributes, and which do not , may help refine non clinical or clinical supporting data needs. Differences need to be thoroughly investigated.
- Provide confidence in the quality , safety and efficacy of rDNA derived biotherapeutics, biosimilars and in the extrapolation of indications, as well as post approval manufacturing changes.
- Confidence in routine lot release testing – choice of relevant quality attributes

# Present Situation and Future Perspectives

- Increasing complexity of licensed biosimilars – insulin 2014 (6KDa): somatropin 2006 (21kDa): Epoetin 2007 (34 kDa); Infliximab 2013 (144.2 kDa)
- Development and production of biotherapeutics /biosimilars expected to expand in Low and Middle Income Countries (LMIC)
- New manufacturers with less experience of biotherapeutics
- NRAs with less expertise / experience and capacity to evaluate these products
- **Less experienced NRAs will become the "first regulatory entry point" for some complex biotherapeutics**
- Many are seeking support from WHO or other NRAs

# 67th World Health Assembly 2014

- First-ever Resolution on biotherapeutics (BTPs) (WHA 67.2) “Access to BTPs including similar biotherapeutic products (SBPs) and ensuring their Quality , Safety and Efficacy”
- Recognizes importance of biotherapeutics for global health
- **Requests Member States** to develop the necessary **scientific expertise** to facilitate development of solid, scientifically-based regulations and **strengthen** national regulatory assessment and authorization frameworks
- **Requests WHO** to support the development of national regulatory frameworks that promote access to quality, safe, efficacious and affordable BTPs, including SBPs and **to update the 2009 biosimilar guidelines taking account of technological advances** for the characterization of BTPs

# Future

- Issues of genetic stability and virus contamination are examples of real issues related to expression systems – here to stay , need careful oversight
- rDNA protein purification and characterization key
- As new inexperienced manufacturers come into operation it is essential that they understand the need for great care and attention regarding development, production and clinical evaluation of rDNA derived products
- Role of NRA in overseeing these developments is critical
- Development and on-going production rDNA products with consistent quality and clinical performance is **NOT EASY**

# Future is bright

- rDNA proteins are playing a major and expanding role in today's medical practice and will do so in the future, especially in tackling unmet clinical needs

- With increasing understanding of structure - function relationships in recombinant protein products we will be better placed to build safe and effective second and third generation recombinant products.

- **There is also the promise of more predictable criteria for licensing and regulatory oversight of these scientifically challenging products.**

# THANK YOU FOR YOUR ATTENTION

Some useful references to follow

# References - genetic stability


- Galibert F (1990) Stability of a gene recombinant: what does it mean and how to check for it? Biologicals 18, 221 – 224
- Corran,P.H., Griffiths E , Robertson, J.S. Geisow M.J (1993) Harmonizing viewpoints on ensuring the consistency and stability of rDNA derived biologicals, Trends in Biotechnology, 11,77-80
- WHO/NIBSC Workshop on genetic stability and product consistency of rDNA derived biologicals, Biologicals 21 (1993) 89-155
- Scorer C A , Carrier, M J, and Rosenberger R F (1991) Amino acid misincorporation during high-level expression of mouse epidermal growth factor in *Escherichia coli.* Nucl. Acid Res 19 3511-3576
- Genetic Stability and Recombinant Product Consistency, (International Association of Biological Standardization Symposium, Annecy, France 1993) in Developments in Biological Standardization Vol 83 , 1994

# References - viral contamination


- Zhan D, et al (2002) Detection of minute virus of mice using real time quantitative PCR in assessment of virus clearance during the purification of mammalian cell substrate derived biotherapeutics, Biologicals 4, 259-270
- Garnick RL, (1996) Experience with viral contamination in cell cultures Dev Biol Stand 88 49-56
- June 2009 Press Release from Genzyme
- Nature Editorial (2011) Pharmaceutical firms should come clean to tackle drug contamination, Nature 472, 389-390

# References - WHO Guidelines / Recommendations

- WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks, Annex 3 , WHO Expert Committee on Biological Standardization. Sixty-first report. Geneva, World Health Organization, 2013; WHO Technical Report Series, No. 978.
- WHO Guidelines on the quality, safety and efficacy of biotherapeutic protein products prepared by recombinant DNA technology. Annex 4 in: WHO Expert Committee on Biological Standardization. Sixty-fourth report. Geneva: World Health Organization; 2014: (WHO Technical Report Series, No. 987)
- Regulatory assessment of approved rDNA-derived Biotherapeutics, in WHO Expert Committee on Biological Standardization. Sixty-sixth report. Geneva, World Health Organization, 2015 (WHO Technical Report Series, No. 999)

# References - WHO Guidelines / Recommendations


- WHO Guidelines on evaluation of similar biotherapeutic products (SBPs). (2009) Annex 2 in: WHO Expert Committee on Biological Standardization: Sixtieth report. Geneva: World Health Organization; 2013 (WHO Technical Report Series, No. 977)
- WHO Guidelines on evaluation of monoclonal antibodies as similar biotherapeutic products (SBPs). Annex 6 in: WHO Expert Committee on Biological Standardization. Sixty-seventh report. Geneva, World Health Organization, 2016 (WHO Technical Report Series, No. 1004).
- WHO Guidelines on procedures and data requirements for changes to approved biotheraeputic products ( in preparation for WHO Expert Committee on Biological Standardization 2017)