

# Chapter 8

## The Biologics Revolution and Endotoxin Test Concerns



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**Abstract** The advent of “*at will*” production of biologics in lieu of harvesting animal proteins (i.e. insulin) or human cadaver proteins (i.e. growth hormone) has revolutionized the treatment of disease. While the fruits of the biotechnology revolution are widely acknowledged, the realization of the differences in the means of production and changes in the manner of control of potential impurities and contaminants in regard to the new versus the old are less widely appreciated. This chapter is an overview of the biologics revolution in terms of the rigors of manufacturing required to produce them, their mechanism of action, and caveats of endotoxin control. It is a continuation of the previous chapter that established a basic background knowledge of adaptive immune principles necessary to understand the mode of action of both disease causation and biologics therapeutic treatment via immune modulation.

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## 8.1 Part I Biologics Overview

### 8.1.1 Introduction

The manufacture and therapeutic application of biologics presents a complex endeavor. Microbiological control personnel need some primer on the complex subject of biologics which is not readily available from a single source. Biologics mode of action is based upon immune modulation and manufacturing has diverged from the more basic historical disciplines to become much more highly specialized. This forms somewhat of an inherent tension in that we need some immunological background to understand changing precepts affecting microbiological contamination control (MCC), yet as non-immunology-expert specialists we cannot expect to obtain an expert understanding of concepts that have exploded in complexity the last ten years.

*Biologics are not just a continuation of large volume parenterals (LVPs) or small molecule drugs (SMDs).* There are many significant differences between biologics and LVPs and SMDs. LVPs are among the oldest injection therapies containing only simple ingredients such as salts, sugars, electrolytes, etc. They generally have no active ingredient whereas, typically, a SMD contains a chemically synthesized active pharmaceutical ingredient (API). Modern biologics are genetically-engineered (cloned from human genes or other natural sources) and produced as recombinant proteins, sugars, or nucleic acids (or complex combinations of these), but also may be whole cells, tissues, blood components, antibodies, etc. The differences that have accrued in terms of both the molecules and the means of production is overviewed here.

### 8.1.2 Differences in Biologics Versus LVPs and SMDs

#### 8.1.2.1 Large, Complex and Produced in Living Organisms

Biologics are large and complex molecules (especially monoclonals as shown in Fig. 8.1) and thus must be produced in living expression systems (bacterial, yeast, plant, mammalian, etc.). They are too complex to be chemically synthesized and are grown up in a series of cell culture tanks. Each of the quotes below focuses on a specific manner in which biologics differ from SMDs.

According to Ganellin, Jefferis, and Roberts: [2]

One has only to consider the size of biomolecular drugs to recognize that the technologies that give rise to biomolecular drugs must be considerably different from the classical SMDs. Genentech equates the difference between aspirin (21 atoms) and an antibody (~25,000 atoms) to the difference in weight between a bicycle (~20 lbs) and a business jet (~30,000 lbs.) [3].

Let us consider how they differ with respect to distribution, metabolism, serum half-life, typical dosing regimen, toxicity, species reactivity, antigenicity, clearance mechanisms, and drug-drug interaction (especially SMD/biologic drug interaction).

According to Baldo: [4]

In comparison to small molecule drugs (SMDs) that are chemically synthesized, biologics are large, complex and not easily completely characterized (i.e. contain structural heterogeneity).

According to FDA: [5]

They (biologics) are also "...heat sensitive and susceptible to microbial contamination. Therefore, it is necessary to use aseptic principles from initial manufacturing steps, which is also in contrast to most conventional drugs".

According to Geigert: [6]

The three-major differences between biologics and "chemical drugs" are:

(1) use of living source materials to produce the biologic, (2) increased complexity of biologic manufacturing processes, and (3) increased complexity of the biologic molecules themselves.

According to USP: [7]

Biologics, or large molecule medicines, are complex in nature and often produced through living expression systems while their pre-existing, small molecule counterparts are chemically defined molecules often produced through chemical synthesis. As the pharmaceutical industry has shifted from producing predominantly small molecule drugs to manufacturing a plethora of large molecule drugs, manufacturers have not only had to adapt existing research tools, production processes, and analytical methods, but also have had to develop novel technologies and approaches. As a result, the quality standards are also evolving to suit the new paradigm of scientific complexities presented by revolutionary biologic therapeutics.

For a more detailed overview of the relative size of various biologic molecules including mAbs and vaccines (virus-like particles and outer membrane vesicles) relative to various whole cell types see van der Pol, Stork and van der Ley [8].

### 8.1.2.2 Produced via Recombinant Methods

Biologics are older as a class than many realize (see Chap. 2) as they began with the development of vaccines as early as 1885 when Pasteur/Roux developed a vaccine for rabies. Jenner's smallpox treatment was used as early as 1798 (and was likely used even earlier by the Chinese) but was not based upon the paradigm of a manufactured or "manufacturable" product, but rather upon the observation that milk maids whom routinely caught cow pox did not seem to get small pox and thus the pus from such sores could be used as preventative inoculates. Pasteur began to understand, develop and apply the specific scientific principles of "*germ theory*". Prior to developing his widely-publicized vaccine for rabies, Pasteur had worked on vaccines for chicken cholera (1878) and anthrax (1881). The Pasteur story is

particularly compelling as he saved a young French boy's life (Joseph Meister) upon the first use of the vaccine in humans.

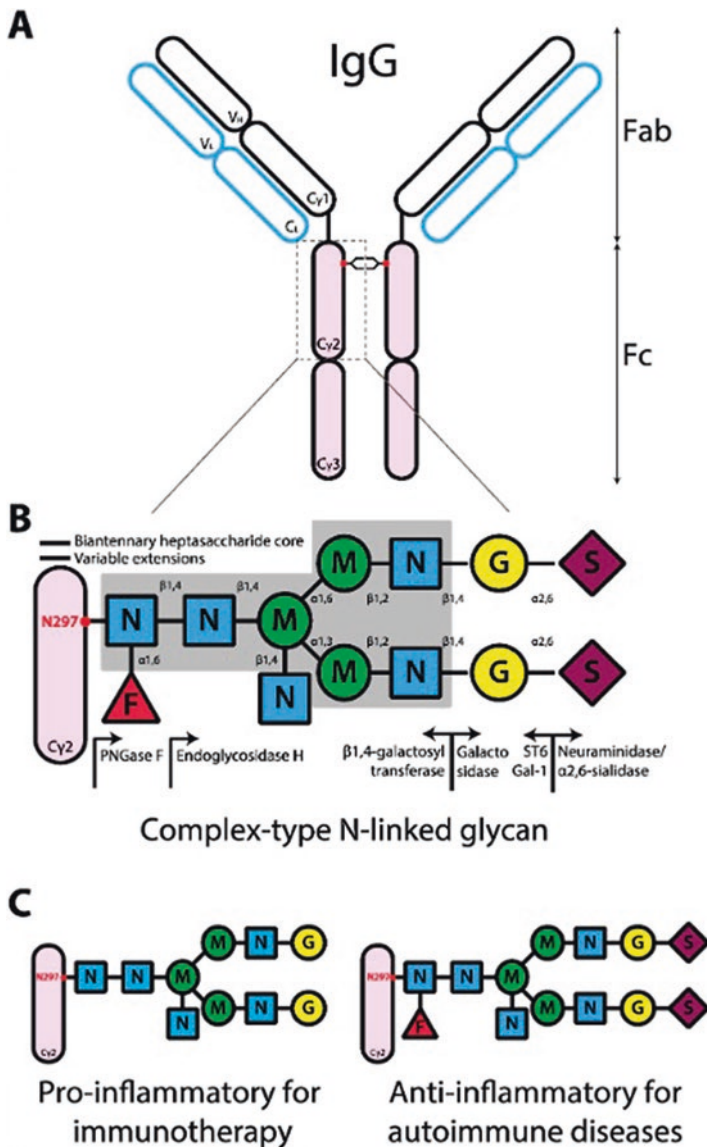
In years subsequent to the first vaccine a surprising number of vaccines were developed for some of mankind's worst diseases including: diphtheria (1888), plague (1897), tetanus (1924), tuberculosis (1927), yellow fever (1936), measles (1963), mumps (1967), and rubella (1969) [9]. Some early efforts show that there were legitimate treatments developed to harness the power of the mammalian immune system via the use of animal serum, antitoxins, and vaccines at a level of sophistication that is surprising given the state of scientific knowledge at the time. While these kinds of early preparations are "biologics" (as derived from living cells), they are not what we think of today by the term "Biologics". Biologics in the modern sense are a class of therapeutics produced by *recombinant DNA technology* where a gene is inserted into a cell culture organism (typically mammalian or microbe) or other growth expression system (plant or transgenic animal). The molecules thusly produced fall into several different classes (see Sect. 8.1.2.6).

Biologics that are truly the result of the biotechnology revolution began in 1982 when Genentech licensed recombinant Human Insulin (rHI) to Eli Lilly for production. Production of recombinant Human Growth Hormone (rHGH) followed in 1985. At 5808 Daltons (51 amino acids) rHI is closer to a peptide drug rather than the size and complexity of modern biologics, especially as compared to monoclonal antibodies which are around 150,000 Daltons (Fig. 8.1). HGH is also a rather small biologic at a molecular weight of 22,124 Daltons (191 amino acids).

As a window into the *power of the biotechnological techniques* (Fig. 8.2) consider the advent of rHI and the subsequent changes to the structure of the insulin molecule used to meet the needs of various dosing regimens. The protein sequence has been tweaked to create fast acting, short acting, long acting, and intermediate acting regimens [10]. As a drug, as opposed to the natural, internal secretion and absorption that occurs constantly inside the healthy human body, insulin, has several challenges including the mode of administration, dosage and timing (relative to food intake and exercise, stress or illness), uptake variability from the blood stream, lack of excised C-peptide in dosed form, etc. Structural variants are called *analogs* and recombinant technology has allowed the production of several variant amino acid structures as detailed below in Fig. 8.3. Without biotechnology one simply could not gain the kind of control over nature's molecules that has been obtained today, even to the extent of man-made evolution in tailoring them to offset the effects of external drug delivery. One cannot look at the "simple" insulin sequence as shown referenced here [11] without gaining a sense of the immense complexity inherent in even very simple protein structures.

### 8.1.2.3 Adverse Responses

The level of knowledge and pinpoint control of protein structure and thus function as represented above in is truly a revolutionary platform for clarifying disease causation, as well as developing treatments and cures. However, biologics side-effects,



**Fig. 8.1** Schematic representation of the human IgG structure and glycan composition. (a) IgG structure. IgG protein is comprised of two heavy chains (black outline) and two light chains (blue outline). Each IgG heavy chain has the variable region (VH) and the constant region containing three domains (C<sub>H</sub>1–3). The line between C<sub>H</sub>1 and C<sub>H</sub>2 represents the hinge region. Each light chain has variable (VL) and constant regions (CL). IgG molecule can be divided into antigen-binding fragment (Fab; empty ovals) and fragment crystallizable region (Fc; pink ovals). The red dot represents N-linked glycans of complex-type. (b) Composition of complex-type N-linked glycan on IgG. The glycan has a biantennary heptasaccharide core (solid line and in the gray block) and variable extensions (dash line). Abbreviations: F fucose, N GlcNAc, M mannose, G galactose, S sialic acid. The enzymes, glycosyltransferases (left arrow) and glycosidases (right arrow), responsible for the addition or removal of the specific sugar are placed directly underneath of the sugar linkage. (From Kai-Ting C. Shade and Robert M. Anthony. CC BY 3.0 [1])



#### 8.1.2.4 The Process Is the Product

Early on in biologics production, manufacturers began to see that manufacturing quality would have to be improved from the processes inherited from LVP and SMD manufacture. With biologics came many additional concerns [14], associated with what had previously been routine processing, including protein aggregation [15, 16], siliconization of stoppers [17], coating of vials [18], the existence of particles down to the subvisible range [19], etc. The highly defined conditions under which biologics must be produced has led to the assertion that, for biologics, “*the process is the product*”. In addition to process impurities arising from the product expression system which must be removed, it is also true for minute differences (heterogeneities) in the product protein structure itself, especially in protein glycosylation [20, 21].

Therefore, for biologics, “*the product is the process.*” Because the finished product cannot be fully characterized in the laboratory, manufacturers must ensure product consistency, quality, and purity by ensuring that the manufacturing process remains substantially the same over time. By contrast, a drug manufacturer can change the manufacturing process extensively and analyze the finished product to establish that it is the same as before the manufacturing change.

The living systems used to produce biologics can be sensitive to very minor changes in the manufacturing process. Small process differences can significantly affect the nature of the finished biologic and, most importantly, the way it functions in the body. To ensure that a manufacturing process remains the same over time, biologics manufacturers must tightly control the source and nature of starting materials, and consistently employ hundreds of process controls that assure predictable manufacturing outcomes [22].

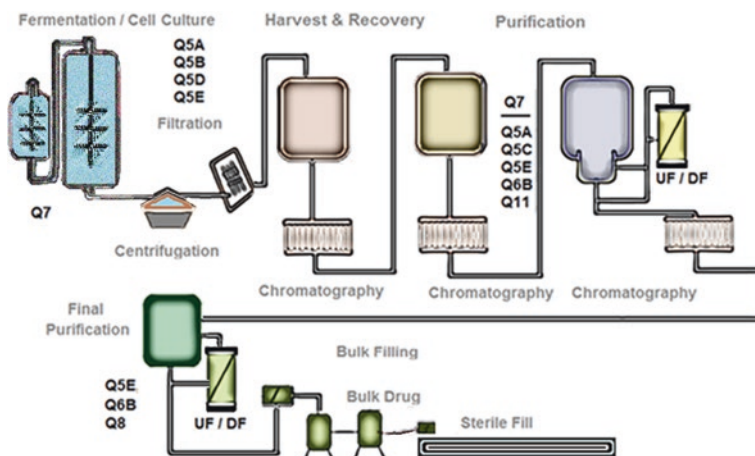
Overt differences exist between SMD manufacturing based on chemical synthesis (e.g. gemcitabine at MW 263 or cisplatin, MW 300) and growth-based biologics production (e.g. Trastuzumab at MW 145,532 or *Adalimumab* at MW 144,190). The modes of action of these four drugs will be briefly compared below. Figure 8.4 shows a simplified manufacturing process flow for a common biologic, a monoclonal antibody, and associated ICH quality requirements.

Due to their complex nature and the limited clinical experience with biologics such as chimeric fusion proteins and biosimilars before approval, a high level of characterization is demanded for their continued development. This characterization needs to cover protein and peptide mapping, and glycan analyses utilizing state-of-the-art analytical methods for the characterization of glycoforms. Liquid chromatography-mass spectrometry (MS) and capillary electrophoresis-MS, as well as classical electrophoretic and chromatographic methods, are playing an increasingly important role in this respect (Baldo, 2017).

The process control of biologics, as they are complex and heat labile (cannot be terminally sterilized), consists of non-sterile processes that are growth promoting upstream as well as pH neutral downstream. Care is taken to control microbial ingress via bioburden and endotoxin monitoring. This has been overviewed by the BioPhorum Operations Group (BPOG) in some detail.

The biopharmaceutical industry produces non-sterile bulk biologics (i.e. Drug Substances) using bioburden controlled processes in accordance to Q7A and Annex 2. Sterile final





**Fig. 8.4** Typical biologic (mAb) manufacturing process flow with associated ICH guidance document references. (Figure derived from Conner 2013 [23], and Davis 2017 [24]). Once the molecule is manufactured it may consist of a subtle heterogeneous mixture of proteins, especially those on the cutting edge of new processes and new therapeutic indications

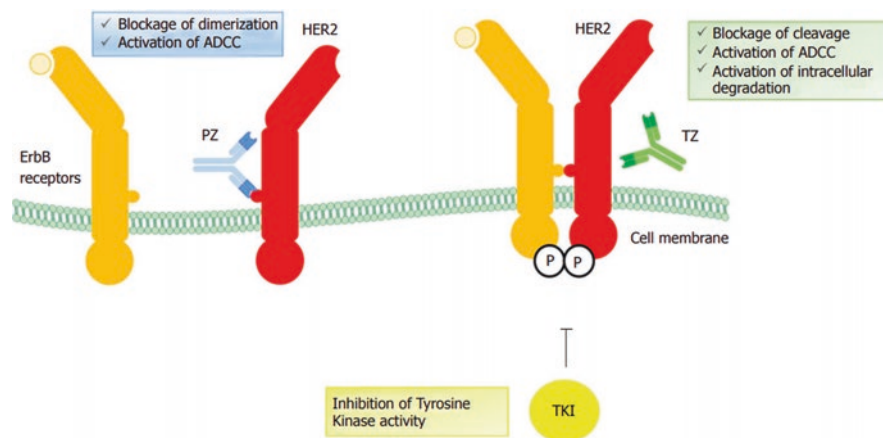
dosage forms are produced in accordance to Annex 1. A mammalian cell mAb process consists of upstream and downstream processes. Upstream operations include the protein production phase of manufacturing where the host cells are grown to generate the product molecule. Primary recovery (centrifugation and depth filtration) is the first step in removing the unwanted production components while retaining the product molecule. Capture of the target molecule is often achieved with affinity chromatography [25].

### 8.1.2.5 Based Upon Immune Modulation

Hand in hand with the biotechnology revolution, the mechanism of action of many biologics is based upon immunological science that has exploded in the last 20 years. When one considers Janeway's 1989 proposition of the existence of pattern recognition receptors (PRRs) as molecular immune receptors to fit microbial PAMPs,<sup>1</sup> then the explosion of knowledge that has come since in terms of the myriad of receptors that control and affect immune interactions becomes apparent. The importance of the elaboration of cell surface signaling molecules (CSSMs), particularly those important to modulating immune responses, in conjunction with the development, manufacture, and use of biologics molecules cannot be overstated. The therapeutic targeting of CSSMs as combined with the development of monoclonal antibodies from hybridoma technology by Kohler and Milstein [26] in 1975, and the continued elaboration of antibody paratopes to fit various antigen epitopes, including human receptors, has, effectively, brought to fruition Paul Ehrlich's original "magic bullet" concept [27].

<sup>1</sup>PAMPs and MAMPs are often used interchangeably as "pathogen" associated molecular patterns versus "microbial" associated molecular patterns as both are used to describe microbial structures that are not limited to pathogens.





**Fig. 8.5** Schematic representation of mechanism of action of HER2-targeted drugs. PZ recognizes an epitope within the HER2 dimerization domain, thus preventing interaction with other activated ErbB receptors. Moreover PZ recruits natural killer cells, which mediate ADCC. TKI act on HER2 tyrosine kinase activity, by blocking intracellular signaling. TZ binds the juxtamembrane portion of HER2, thus preventing receptor cleavage and stimulating ADCC response and receptor degradation after endocytosis of the HER2-TZ complex. PZ Pertuzumab, TZ Trastuzumab, TKI Tyrosine kinase inhibitors, ADCC Antibody dependent cellular cytotoxicity, HER Human epidermal growth factor receptor, ErbB erythroblastic leukemia viral oncogene homolog [30]

The detailed knowledge of the working mechanisms of large, immune-active biomolecules such as monoclonal antibodies is integral to understanding their function as “magic bullets”. The interaction of therapeutic biomolecules with CSSMs is only beginning to affect the way microbiological control is viewed. At present, this difference is not largely appreciated as a paradigm shift. Manipulation of CSSMs is therapeutic control at the sub-cellular, molecular, and almost atomic level in terms of disease treatment. Biologic therapeutic action is often gained by the interaction of protein ligands and receptors as CSSMs. A lymphocyte has more than 300 CSSMs on its surface including TLRs and, all in all, based upon human genome project sequence annotation, “more than 4,000 molecules have been identified as potential CSSMs, based on similarities in their transmembrane protein structure, along with their signature intracellular and extracellular domains” (Zhu, Yao, and Chen). Refer back to Fig. 7.12.

The difference in biologics and SMDs is exemplified in comparing the mechanisms of action of two small molecule cancer treatment compounds-gemcitabine and cisplatin-with two biologics (mAbs), Pertuzumab and Trastuzumab, the former of the two biologics prevents “ligand induced dimerization” and the later disrupts “ligand-independent complex formation” (Baldo, 2017). See Fig. 8.5. MAb are large molecules that specifically target CSSMs to either prevent or promote (co-stimulatory/co-inhibitory) the fulfillment of functions important to specific cancers (in this instance). The mode of action of the biologics contrasts the small molecules

that enter into cells and thus largely target fast growing tissues. Gemcitabine is a nucleotide analog that mimics cysteine (the C in DNA/ATGC) but curtails replication due to imperfect mimicking of nucleotide action and cisplatin has a different mode of action but similarly disrupts DNA replication. Since cancer cells are the fastest growing cells (and apparently for no better reason), then more cancer cells die than normal cells. Thus, the SMD cancer treatments are more invasive (entering not just cancer cells but also healthy cells), non-specific (no specific molecular target other than the DNA common to all cells), prone to generate resistance over time (limited window of positive effects), and generally bring more unpleasant side effects than the new biologic drugs. According to Barker and Andrews: [28]

This results in side effects which have a significant impact on the patient, such as gut toxicity, immunosuppression and hair loss; these effects limit the doses of chemotherapeutic that can be given and the overall efficacy that can be achieved in many situations. Thus, whilst cytotoxic chemotherapy is useful in some types of tumours, the results for many of the common solid tumours are relatively disappointing and agents that are effective against these solid tumours and which are better tolerated would represent a major step forward in the fight against cancer.<sup>2</sup>

The advent of biologics (especially mAbs) that bind to various receptors (CSSMs) thought to be active in cancer progression has greatly improved therapy options for a number of cancer types, including targeting HER2 for breast cancer. Note that above the relative size of the target receptor and the mAb is graphically represented as widely different whereas the molecule sizes are not so dissimilar (~150 kDa and 185 kDa respectively).

The human epidermal growth factor receptor 2 (HER2, ErbB2) is a 185-kDa transmembrane glycoprotein receptor tyrosine kinase involved in the signal transduction pathways leading to cell growth and differentiation. In contrast to the other three members of the epidermal growth factor receptor family, HER2 is thought to be an orphan receptor, i.e. lacking a ligand. However, HER2 forms heterodimers with any of the related receptors, resulting in receptor activation. Enhanced levels of HER2 have been shown to correlate with one form of aggressive breast cancer, precipitating the development of the HER2-binding monoclonal antibodies (mAb) trastuzumab and pertuzumab [29].

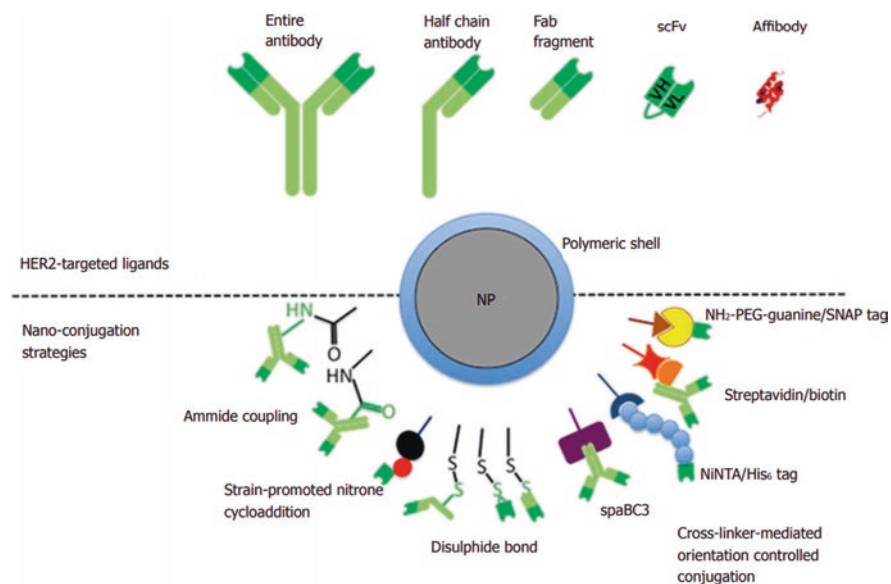
To take Fig. 8.5 one step further, Mazzucchelli et al. graphically demonstrate the development of additional treatments in the form of truncated immune modulating sequences as attached to nanoparticles. In the top part of Fig. 8.6, an emerging trend of utilizing, when possible, only the active part of the antibody is demonstrated (left to right). Also being explored is the use of VLRs<sup>3</sup> as therapeutic candidates [31].

The main takeaways from the figures above relevant to this discussion are (a) the incredible specificity, (b) various targeting possibilities, and (c) the future-oriented development of novel methods of immune modulation aimed at specific receptors and receptor moieties.

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<sup>2</sup>Note that Chap. 7 showed some of the progress being made.

<sup>3</sup>Variable lymphocyte receptors (VLRs) are derived from lampreys as intermediate immune molecular forms between invertebrate innate only animals and jawed vertebrates which have adaptive immune systems.



**Fig. 8.6** Schematic representation of HER2-targeting ligands and conjugation strategies employed for NPs functionalization. HER Human epidermal growth factor receptor, scFv Single-chain fragment variable antibodies (Mazzuchelli et al. [30])

### 8.1.2.6 Biologics Drug Classes

As an overview of various classes of biologics, seven major classes (with enzymes, hormones and peptide drugs combined into a single category) are described below and include an example drug and mechanism of action (MOA) description.

1. **Monoclonal Antibodies (mAbs)**- mAbs consist of three major classes that include (a) immune stimulating (i.e. cancer treatments), (b) inflammation and autoimmune suppressing and (c) miscellaneous, a basket of molecules that includes several novel acting drugs for the treatment of asthma, anthrax, organ transplant and RSV infection (Baldo). Omalizumab is an asthma drug that acts by binding to IgE antibodies in the Cε3 paratope region that is important for IgE to bind mast cells. In this way, Omalizumab interferes (via steric hindrance) with mast cell activation which includes the reduction of adverse allergic type responses including anaphylaxis. The suppression of the immune system in some specific biologic treatments against autoimmune diseases has also lead to the very slightly increased occurrence of a rare brain cancer caused by a usually latent virus [32].
2. **Enzymes, hormones, glycoprotein hormones, and peptide drugs**- human enzyme deficiencies include those from genetic disorders (inborn errors of metabolism) and those with levels (too much or too little) affected by other disorders. Certain genetically acquired disorders can be corrected by supplying

enzymes that have (via recombinant production) corrected receptors (CSSMs), for example, as described below.

The discoveries of specific recognition of phosphorylated mannose residues, mannose receptor-mediated uptake of lysosomal enzymes, and the presence of these receptors on macrophages, demonstrated that a lysosomal enzyme needs to be specifically recognized by its target cells. These insights led to the first successful enzyme replacement therapy (ERT) for type I Gaucher disease that occurs with a frequency of 1 in 75,000 births worldwide, making it the most prevalent of the sphingolipid storage disorders. Gaucher disease is the result of an inborn error of metabolism due to a deficiency of the lysosomal acid  $\beta$ -glucosidase glycoprotein,  $\beta$ -glucocerebrosidase (glucosylceramidase;  $\beta$ -glucosyl-*N*-acylsphingosine glucohydrolase) which cleaves  $\beta$ -*D*-glucosylceramide (glucocerebroside) into glucose and ceramide. The enzyme's substrate is a widely distributed cell membrane component and, in the absence of  $\beta$ -glucocerebrosidase, glucocerebroside, and other glycolipids accumulate by as much as 20–100-fold in the lysosomes of cells, particularly macrophages and other cells of the reticuloendothelial system. With this background, glucocerebrosidase prepared from human placenta and marketed as Ceredase® was used to reverse the clinical manifestations of type I Gaucher disease by targeting the patients' macrophages after sequential deglycosylation to expose mannose residues. Imiglucerase, a recombinant, deglycosylated glucocerebrosidase prepared in Chinese hamster ovary cells by DNA technology, was soon introduced and because it proved at least as clinically effective as Ceredase® and could provide a pathogen-free preparation in almost unlimited amounts, it (as Cerezyme®) soon replaced its predecessor. (Baldo)

This example, along with the switch from animal-derived insulin to recombinant human insulin, shows the vast utility of recombinant proteins (r-proteins) as compared to harvesting natural forms (n-proteins). Hormones and peptide drugs include insulin, glucagon, human growth hormone, vasopressin, and parathyroid hormone. Glycoprotein hormones include follicle-stimulating hormone and thyroid stimulating hormone. As an aside, the use of r-proteins such as human insulin brings a greater exactness and specificity to treatments whereas reliance upon animal extracted proteins was time consuming, laborious and made for less desirable drugs (especially from a microbiological and adventitious contaminant control vantage).

3. **Vaccines** are harmless biological preparations made from infectious agents intended to stimulate the immune system to produce a long-lasting immune protection. Some important diseases remain that cannot be protected against via vaccination including AIDS, tuberculosis, malaria, and prion disease. Cancer vaccines are also being developed. MAbs are being developed as an immune treatment (as an alternative to long-acting vaccination) for some infections (as in RSV).<sup>4</sup> Subunit vaccines are viewed as safer in that a protein gene cloned and expressed for use as a specific antigen cannot revert to a disease-causing state as can an attenuated organism. An example of this is the hepatitis B vaccine that uses a surface protein to aid in the prevention of hepatitis associated liver cancer. DNA vaccines have also been developed to encode a specific antigen protein after injection. Interestingly, for DNA vaccines, CpG bacterial sequences are included as the adjuvant type. This speaks to the modern use of TLR activators

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<sup>4</sup>In Chap. 2, the ineffective formaldehyde treatment of a diphtheria toxin resulted in disease from the vaccine.

(PAMPs) as adjuvants and parallels the inclusion concerns of impurity/contaminants as adjuvants.

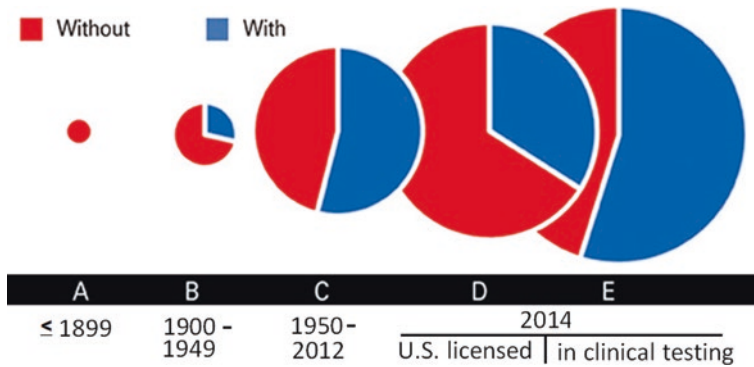
Beyond doubt, the advent of sequencing, rendering microbial genomes readily accessible, has been of utmost importance for vaccinology and has allowed for highly rational identification of vaccine antigens. Many novel vaccines currently in development are thus based on proteins or peptides predicted by computer databases or by screening antigen libraries [33, 34]. Although these advances in the post-genomic era have enabled the design of highly pure, safe and simple vaccines, other challenges have emerged in parallel, including the inherent lack of immunostimulatory properties of proteins and peptides. Vaccine adjuvants are therefore considered key components in modern vaccinology since they provide the necessary help of enhancing the immune responses [35]

Here one may have picked up on an inherent contradiction. That is, that foreign proteins from microbes and virions produced recombinantly and purified are often not immunogenic and thus adjuvants (that are in essence contaminants) must be added whereas purified human recombinant proteins (such as based on IgG scaffold) manufactured as recombinant proteins often come with immunologic side effects.

Early vaccines had no need for adjuvants, as they contained everything but the kitchen sink, microbiologically speaking. As vaccines have become more purified, they have become less immunogenic. Unwanted side effects may come from using killed whole microorganisms or from adding too ambiguous an immune irritant. Therefore, today the choice of an adjuvant is a sophisticated effort. Contained in that effort is also the attempt to stimulate the appropriate immune response pathway to match the specific immune mechanism of action of the disease which is being vaccinated against (see Fig. 8.7).

Very few antigens are inherently immunogenic and virtually all vaccines require adjuvants in some form, endogenous or exogenous. Without a component that engages either innate immune cells or additional receptors on lymphocytes such as complement receptors [36], most non-adjuvanted, highly-purified antigens induce tolerance rather than immunity [37]. Very few antigens, such as certain toxins, are capable of inducing antibody responses when administered without adjuvants. Because of their immunogenicity, non-toxic derivatives of some toxins are being developed as adjuvants themselves, such as cholera toxin (CT) or *E. coli* enterotoxin (LT) (reviewed in [38]). The first scientific reports of exogenous adjuvants deliberately added to vaccines are less than a century old and come from Gaston Ramon in the 1920s [39]. The substances he added to vaccines to “enhance immune responses” were complex and poorly defined and included tapioca starch and agarose. These early adjuvants, however, did trigger inflammation, which subsequently enhanced vaccine-specific lymphocyte responses. Adjuvanticity in this scenario is through a bystander effect with a significant amount of “wasted inflammation” (Quote from N.M. Valiante (Novartis Vaccines)), defined as excessive innate immune responses, which result in reactogenicity but only partially contribute to the adaptive immune response [40].

4. **Coagulation factors** – mammalian blood coagulation is a complex process with the interaction of many factors (and contrasts the greatly simplified *Limulus* coagulation process). Queen Victoria (born in 1819) had hemophilia and thus the disease had been known long before the blood system factors were characterized. There are two types -*hemophilia A*, which occurs due to the lack of clotting factor VIII, and *hemophilia B*, which occurs due to the lack of clotting factor IX. Both afflictions are typically inherited from one’s parents through the X

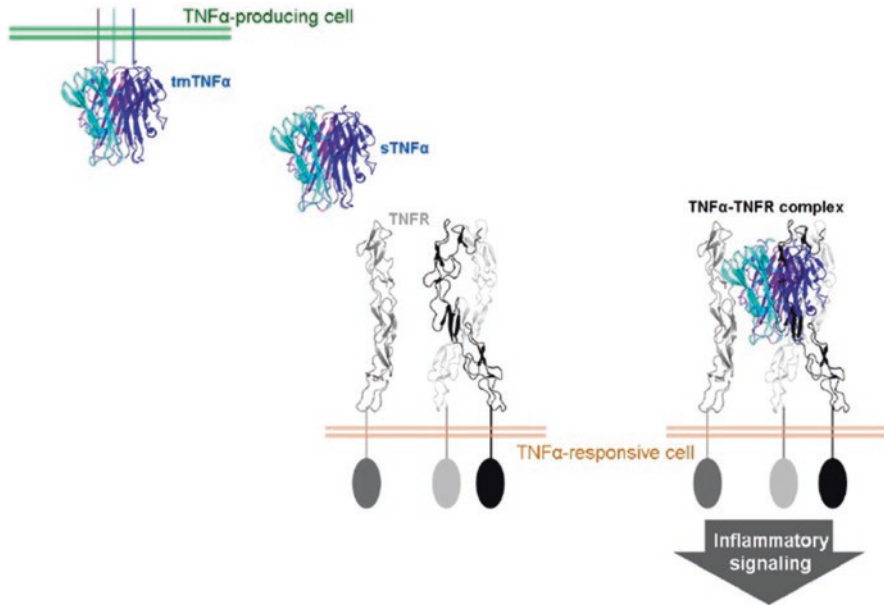


**Fig. 8.7** Proportion of human vaccines containing adjuvant through stages of history. Circles depict periods in vaccine development with fractional amount containing adjuvant shaded in blue, and diameter proportional to the log of number of different vaccines. A, up to 1899; B, 1900 to 1949; C, 1950 to 2012; D, 2014 U.S. licensed as listed by Food and Development Administration (FDA); E, 2014 in clinical testing as listed by HuVax (<http://www.violinet.org>). Note that the licensed vaccines group D contains many more multiple products for similar or overlapping indications, most of which are non-adjuvanted, while the experimental group E includes all existing and new candidate adjuvants reported in clinical testing. Derived from Powell, Andrianov, and Fusco [41]. (Korean Vaccine Society)

chromosome with a nonfunctional gene and thus disproportionately affect males. There are more than a dozen blood clotting factors produced recombinantly including Factor VIIa, VIII, IX, IX FC (fusion protein), XIII. In addition to recombinant forms there are also many forms purified from pooled human plasma. Some of both the recombinant and natural forms may include severe hypersensitivity and fever responses. The mechanism of supplying a specific enzyme for a clotting pathway is merely to supply the missing link (functional enzyme) that breaks the chain of events (i.e. protease cascade) necessary to properly coagulate the blood.

- 5. Cytokines-** are produced by cells of the immune system and are generally thought of as signals act that locally upon CSSMs versus hormones which act over greater distance in the body, however, systemic effects also result from additive local effects from cytokines (including sepsis). This category includes both drugs used as cytokines and as cytokine receptor antagonists to modulate the immune response, including chemokines, interferons, interleukins, lymphokines, and tumor necrosis factors (TNF) but not hormones or growth factors (which are another class of biologic therapy). As of June of 2016, there were 23 approved cytokines (and antagonists) as biologics therapeutic drugs, including interferons, interleukins, erythropoietin, bone morphogenic proteins, receptor antagonists for IL, INF and TNF. Anakinra, for example, binds to the IL-1RI receptor thus blocking IL-1 $\alpha$  and  $\beta$ . The IL-1RI /IL1- $\alpha$  and  $\beta$  receptor system is important in governing the activities of cartilage degradation and bone resorption.





**Fig. 8.8** Biology of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ). A soluble TNF $\alpha$  (sTNF $\alpha$ ) trimer is released from its transmembrane form (tmTNF $\alpha$ ) and binds to a preassembled trimer of TNF receptor (TNFR), thereby mediating inflammatory signaling. Each protomer of TNF $\alpha$  homotrimer is colored blue, cyan, and purple. The green and pale red bars indicate membranes of a TNF $\alpha$ -producing and TNF $\alpha$ -responsive cells, respectively (From Lim et al. [43])

An overview of the actions of various cytokines is given by Tisoncik et al. [42]

- **Interferons**-regulation of innate immunity, activation of antiviral properties, anti-proliferative effects
  - **Interleukins**-growth and differentiation of leukocyte recruitment; many are proinflammatory
  - **Chemokines**-control of chemotaxis, leukocyte recruitment; many are proinflammatory
  - **Colony-stimulating factors**-stimulation of hematopoietic progenitor cell proliferation and differentiation
  - **Tumor necrosis factor**-proinflammatory, activates cytotoxic T lymphocytes.
6. **Fusion or chimeric proteins**- two different proteins, each with a unique function, can be fused to form a single therapeutic protein. Etanercept is a fusion protein that uses the Fc portion of IgG antibody linked to the ligand-binding portion of a TNF receptor. The binding of the fusion molecule to the TNF receptor prevents the interaction of the receptor with the TNF cytokine thus reducing the associated inflammatory responses in rheumatoid arthritis and other autoimmune diseases. TNF receptor binding is shown below in Fig. 8.8.



7. **Cell therapy**- the use of IgG fragments as well as whole immune cells (i.e. T cells) that have been genetically modified consists of those taken from an individual and returned to the same individual (autologous) and those that have been taken from one but returned to many (allogenic). The earliest cell types to be taken and returned were for bone marrow stem cell transplantation. Some promising new cancer treatments are based upon the modification of T cell receptors/ T cells such that they are sensitized to neoplasms. An interesting differentiation between cells grown for administration and those grown to produce a recombinant drug is that the cells for r-production are grown and discarded and are often known to accrue genetic aberrations over time (CHO) as this is not their natural mode of growth (cell culture). However, the introduction of whole cells must contain only cells that maintain the proper genetic structure (“phenotype, genotype and even karyotype”) and must not contain cell culture media [44].

A rapidly emerging immunotherapy approach is called adoptive cell transfer (ACT): collecting and using patients’ own immune cells to treat their cancer. There are several types of ACT (see “ACT: TILs, TCRs, and CARs”), but, thus far, the one that has advanced the furthest in clinical development is called CAR T-cell therapy.

In 2017, two CAR T-cell therapies were approved by the Food and Drug Administration (FDA), one for the treatment of children with acute lymphoblastic leukemia (ALL) and the other for adults with advanced lymphomas. Nevertheless, researchers caution that, in many respects, it’s still early days for CAR T cells and other forms of ACT, including questions about whether they will ever be effective against solid tumors like breast and colorectal cancer [45].

### 8.1.2.7 Clinical, Regulatory, Legal and Manufacturing Cost Differences

Biologics present some special instances regarding studying the effects of biomolecules in pre-clinical and clinical studies. The following relevant categories are listed by Kingham et al. [46]:

- **Relevant species**- often biologics display reactions that are specific to various non-human species and therefore must be studied in the most relevant species, some of which are not always the more common ones. This has also been seen to extend to tissue specificity.
- **Immunogenicity**- the common elicitation of immune responses and effects must be considered for biologic drugs. “Often, their clinical development programs must include an assessment of immunogenicity, which is typically not an issue for small molecule drugs.”
- **Manufacturing process changes are scrutinized even at the clinical stage**, since these drugs are more affected by process changes in manufacturing, FDA will decide whether a sponsor will have to perform additional studies to ensure that such changes are not deleterious to the product.

From a regulatory vantage, biologic drugs **require the submission of a Biologics License Application (BLA)** whereas new drugs require a New Drug Application (NDA). The “biologic” designation also **conveys extended legal protection** in terms of intellectual property rights.

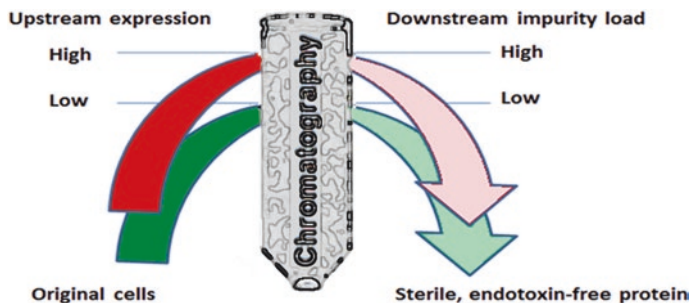
In the United States, “biological products” are subject to a different premarket pathway and differing intellectual property protections than products regulated only as “drugs.” Whereas a biological product must be licensed pursuant to a biologics license application (BLA) showing it is “safe, pure, and potent,” the sponsor of a nonbiologic drug must submit a New Drug Application (NDA) showing the drug is safe and effective. Certain new biological products receive 12 years of data protection, but new drugs receive up to 5 years of this protection. Biologic and drug legislation also provide different schemes for resolving patent issues regarding entry of follow-on products. Thus, determining whether a product meets the definition of “biological product” is enormously important (Kingham).

Along with these distinctions, proteins that consist of 40 amino acids or smaller have been classified as “peptide drugs” and not as biologics. According to Timmis [47], “when combined with regulations requiring FDA approval of manufacturing processes and facilities-let alone the actual drug-this complexity ensures a significantly more expensive creation process than is common for traditional chemical drug.” And, “estimates place the cost of creating a manufacturing facility for a new biologic drug, excluding materials, between \$200 and \$400 million... bringing a new biologic to market costs an estimated \$1.2 billion.”

### **8.1.2.8 Require Additional Microbiological Control: Expression System Removal**

It is intuitive that an increase in cell expression increases the yield of the therapeutic protein but also increases the impurities to be removed due to the increased mass of cells. Thus, the weight on chromatography and other removal methods for host cell protein and nucleic acid removal increases. Shown below this dual load (product and cell refuse) sits at the apex of the upstream and downstream process flow (Fig. 8.9).

**Microbial Mimetics** Mimetics are not actual microbiological byproducts or cell constituents but rather process constituents that may be immune activating in the body, as surveilled by the immune system, and treated as if it were of a microbial nature (recurring patterns, for example may mimic virus-like particles from the view of the immune system). Mimetics have been shown to include protein aggregates, emulsions such as silicone coatings for stoppers or vials, and particulates, even subvisible particulates. The control of mimetics was of little concern in the manufacture of LVPs or SMDs other than relatively large particulates.



**Fig. 8.9** Impurity load requiring removal is relative to the density of cell growth and protein expression

**Host Cell Protein (HCP) Impurity Determination** HCP removal is applicable to biologics as produced from living cells. As these cells grow and produce therapeutic proteins, they also produce other substances (simply by being alive, reproducing, etc.) that may include those excreted (exotoxins) as well as those intrinsic to their makeup (DNA, RNA, porins, flagellin, endotoxin, etc.). These residues must be removed via various chromatography steps and may include endotoxin in very large amounts if *E. coli* if used as an expression system. The importance of HCPs to impurity control is given by Wang et al. [48].

HCPs constitute a major group of process-related impurities in a drug product. The risks associated with HCPs are primarily immunogenicity. HCPs are complex mixtures with diverse physiochemical and immunological properties [49]. Almost all HCPs carry clinical safety risks as foreign proteins due to the potential to elicit immune response in humans. In addition, some HCPs can also act as adjuvants to enhance immune response to a drug product [50, 51]. Certain HCPs with proteolytic activity can also affect drug product stability and efficacy if not adequately removed or inactivated [52]. HCPs have the potential to affect both the safety and efficacy aspects of a given drug product.

How are all these non-product proteins detected and, thus, how can it be determined if they have been removed? Typically, polyclonal antibodies are raised against the product and expression system that is manufactured *minus the drug protein* and subsequently, in batch to batch runs, the determined set or library of polyclonal antibodies can be used to determine the level and extent of removal success via ELISA testing. The identification of specific protein markers known or suspected to be immunogenic has also accrued.

**Nucleic Acid Removal** According to Wang et al. [53] biologics production has some unique aspects of concern in the removal of nucleic acids, particularly residual DNA:

Residual DNA (rDNA) is comprised of deoxyribonucleic acid (DNA) fragments and longer length molecules originating from the host organism that may be present in samples from recombinant biological processes. Although similar in basic structural base pair units, rDNA may exist in different sizes and physical forms. Interest in measuring rDNA in recombinant products is based primarily on demonstration of effective purification during manufacturing, but also on some hypothetical concerns that, in rare cases, depending on the host expression system, some DNA sequences may be potentially infectious or oncogenic (e.g., HIV virus and the Ras oncogene, respectively) ....

Genomic DNA from microbial sources, on the other hand, could add to risk of immunogenicity to the target recombinant protein being expressed, due to the high CpG content and unmethylated DNA sequence. For these and other reasons, it is necessary for manufacturers to show clearance of DNA throughout production processes and to confirm low levels in the final drug substance using an appropriately specific and quantitative analytical method.

The most common methodology for rDNA quantitation used currently is real-time polymerase chain reaction (RT-PCR), a robust and proven technology. Like most rDNA quantitation methods, the specificity of RT-PCR is limited by the sequences to which the primers are directed.

**Beta Glucans** Fungal cell wall constituents, especially beta glucans, have come to be considered by many to be innate immune response modulating impurities (IIRMI) and should therefore be removed or precluded from biologics manufacturing processes during process development, as seen in the following efforts:

- Multipronged approach to managing beta-glucan contaminants in the downstream process: control of raw materials and filtration with charge-modified nylon 6,6 membrane filters [54].
- Development of downstream processing to minimize beta-glucan impurities in GMP-manufactured therapeutic antibodies [55].

Note that beta glucans, if not an impurity from fungal residues, have been found to be a by-product of breakdown from cellulosic filters: “They are a potential contaminant in pharmaceutical products, originating from *cellulose*-based *filters* and other raw materials used in pharmaceutical processing. [56]” They also show up as false-positives during endotoxin testing. They represent a “false-positive” result because while  $\beta$ -glucans interfere with BET they cannot be quantitated by BET as BET has no  $\beta$ -glucan standard included. See Chap. 16 for an overview of  $\beta$ -glucan detection products. The rationale for not including them in routine testing, but rather precluding them during process development is that they are unlikely to “crop up” via contamination from fungi whereas endotoxin is from Gram negative bacteria that can easily proliferate. They are much more likely to be present due to either (a) cellulosic filter usage (breakdown of cellulose and such filters can be switched out for non-cellulosic filters) or (b) as a fungal sugar present in a raw material (that can also be precluded).

### 8.1.2.9 Require Additional Microbiological Control: Bioburden and Endotoxin

With the advent of biologics it soon became apparent that there would be different rules for biologics given the propensity for immunological responses. For biologics, given the use of various expression systems as growth-based processes, there is the need for removal of host-cell impurities and the monitoring of non-sterile processes that require the demonstration of hold time studies to prove that process conditions do not allow for microbiological overgrowth. Additionally, there is much that is new in terms of microbiological control strategy. In Chap. 9 Shen describes methods used to remove endotoxin impurities or contaminants. It is good to make the distinction between impurities which are potential residual components of the upstream product expression system (CHO cell, *E. coli*, yeast, plants, etc.) and potential contaminants which arise from downstream non-sterile process ingress (i.e. from bioburden). Both routes can include endotoxin: upstream if the expression system is a Gram-negative organism such as *E. coli* or downstream via water-based/buffer downstream processes as Gram-negatives are the likely forms to grow in non-sterile and pH neutral aqueous solutions.

The issue of bioburden for biologics can be viewed from the compliance perspective as an outcome of specific inspection findings, where, according to some, bioburden rarely happens except when the regulators show up. However, the exceptional proficiencies and capabilities of some manufacturers does not automatically transfer to others (some are better than others). This can be viewed in FDA warning letters [57].

- “Multiple lots of drug substance released which had unacceptable high levels of bioburden during the final purification steps.”
- “No microbial limits are established for processing steps, and results show high bioburden levels (>1000 CFU/mL)”
- “... during the harvest of the unprocessed bulk, sublots of Lot (redacted) and Lot (redacted) exceeded the established action limits for bioburden”

The BPOG recommendation for in-process biologics bioburden for the investigation of materials that are “too numerous to count (TNTC)”, in the specific context of the current discussion, also seems not overly stringent [58].

- “A TNTC result for any in-process bioburden sample should automatically result in an Action Level or Out of Specification (OOS), which requires investigation.”

The costs of a bioburden excursion in biologics manufacture can be substantial. Westman summarizes his estimate of financial impact in Table 8.1.

A significant paradigm change in manufacturing that could have a significant positive impact on MCC includes the use of **single-use bioreactor containers**.

A new trend addresses an implementation of single-use bioreactor systems. They have the advantages of lower capital investment and operational costs, flexibility, improved

**Table 8.1** Potential financial impact of a bioburden incident in biopharmaceutical manufacturing [59]

Issue	Potential impact and cost
Commercial impact	Up to USD 1 billion in lost revenue <sup>a</sup> Loss of reputation by customers, authorities, patients Long lead time due to low inventory Lost business to competitors Penalties in rare cases
Failed production lot/scrap batch	Up to USD 1 million <sup>b</sup>
QA investigation	Up to USD 20,000 <sup>c</sup>
Sanitization of facility and equipment	Up to USD 100,000 <sup>d</sup>
Resin must be discarded	Up to USD 3 million <sup>e</sup>

Assumptions:

<sup>a</sup>Up to USD 1 billion lost revenue/month for blockbuster drugs

<sup>b</sup>2000 L bioreactor with 5 g/L expression level. Cost of mAb production USD 100/g

<sup>c</sup>USD 120/h labor cost. 1 week investigation by three people

<sup>d</sup>USD 120/h labor cost. 4 weeks by five people.

<sup>e</sup>Based on large-scale column size and resin costs

production scheduling and higher process replication. They are applicable for GMP manufacturing and available up to 2000 L in scale. Different designs of single-use reactors are available, such as wave, orbital shaken, pneumatically mixed and stirred tank bioreactors. They eliminate the need for cleaning or sterilization and, thus, significantly reduce contamination rates [60].

For biologics manufacture there are many bioburden/endotoxin sampling expectations. An outline of bioburden and endotoxin sampling expectations for monoclonal antibody and recombinant protein drugs has been given by the BioPhorum Operations Group, or BPOG, in some detail [25].

The biopharmaceutical industry produces non-sterile bulk biologics (i.e. Drug Substances) using bioburden controlled processes in accordance to Q7A and Annex 2. Sterile final dosage forms are produced in accordance to Annex 1. A mammalian cell mAb process consists of upstream and downstream processes. Upstream operations include the protein production phase of manufacturing where the host cells are grown to generate the product molecule. Primary recovery (centrifugation and depth filtration) is the first step in removing the unwanted production components while retaining the product molecule. Capture of the target molecule is often achieved with affinity chromatography...

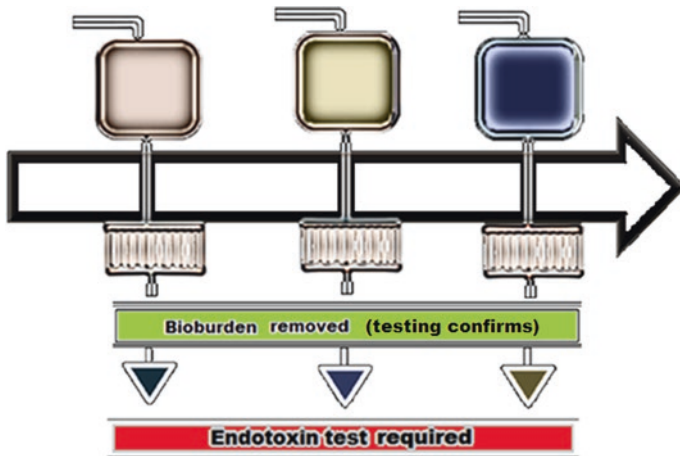
Regarding recommendations arrived at by the BPOG group, the following “talking points” are relevant to MCC in biologics production. Since BPOG is represented by many Pharma companies (16 names on the paper), then it can be considered a current Pharma consensus. The following points are derived from the BioPhorum published document. This is a condensed overview, therefore for specific detail go to the source document. Recommended sampling points include (paraphrased for brevity) the following:

- Media preparation - on an appropriate number of batches at scale, based on statistical analysis and/or risk assessment, test for bioburden/endotoxin just prior to filtration or sterilization of the media
- For filtered buffers, perform bioburden/endotoxin on appropriate number of batches at scale
- For final diafiltration / formulation buffers, endotoxin testing is recommended prior to use...
- Non-filterable buffers should be freshly prepared and used as soon as possible. Bioburden and endotoxin sampling/testing for non-filtered buffers is recommended.
- Prior to transfer to the next bioreactor, a bioburden sample may be tested or kept as a back-up sample...
- Clarified harvest pool (filtered or unfiltered) should be tested for bioburden/endotoxin for all batches just prior to the start of the following process step.
- To evaluate column and UF/DF performance perform bioburden/endotoxin testing on an appropriate number of batches at scale.
- Endotoxin/bioburden test protein pool (filtered or unfiltered) at the end of chromatography and UF/DF operations
- Resin storage conditions should be tested for bioburden.
- Take bioburden and endotoxin samples from the final bulk drug substance (post filtration).
- Plate count recoveries >250 CFU on the most dilute sample are reported as TNTC which for any in-process bioburden sample should result in an Action Level / OOS, which requires an investigation.
- Unlike non-sterile drug manufacturing where the processing environments are often hostile to microbial growth or include the addition of preservatives, manufacturing of biologics require growth mediums (upstream) that enhance bacterial growth or process buffers (downstream).

Perform an assessment when bioburden action level excursions or adverse trends are noted. *Questions that have to be asked include the following:*

- What organism(s) was recovered?
- Is the disinfectant procedure effective at removing the recovered organism?
- What toxins and/or microbial byproducts does the organism(s) produce or release?
- What stage in the process did the excursion occur?
- What downstream purification steps were performed after the organism(s) was recovered?
- Are purification steps validated to remove bioburden? Is there data supporting clearance of possible microbial byproducts?
- Are there connections in other bioburden excursions in upstream or downstream processes?
- Have the same organism(s) been seen with previous lots?
- How long was product held at the step of the excursion and at what temp and pH?





**Fig. 8.10** In downstream processing, bioburden can be filtered out, process step to process step—whereas smaller constituents including and especially endotoxin cannot be due to the size of biological molecules. Thus, processing requires confirmation via *Limulus*-based testing

- Were there changes to the manufacturing process (e.g. equipment) prior to the excursion(s)?
- What potential impact could the recovered organism(s) have on the manufactured protein?
- What is the impact of microbial byproduct production (toxins) on patient / product safety?
- Are all IPC<sup>5</sup> results within historical trends and does the DS meet release specifications?
- What information was gathered during studies conducted during the investigation?

Some have posed the question: since endotoxin comes from bioburden (assuming a non-*E. coli* expression system), then why can't one just test for bioburden and not endotoxin? What is made apparent from the BPOG assessment is that between various process steps filtration of bioburden occurs and thus while removing whole cells, it can leave a cumulative amount of non-viable microbial residue including (and especially) endotoxin. This makes *Limulus*-based testing critical to perform in support of the bioburden results which, may, step to step, begin again from zero in a serial fashion. In this way, *Limulus*-based testing is necessary to perform in conjunction with bioburden testing. This is depicted in Fig. 8.10.

As described above in detail, bioburden and endotoxin routine sampling are a manufacturing expectation. However, in addition, since specific biologic manufacturing processes have specific time (process steps and hold times), temperature and environmental definitions, then these specifically defined parameters must be shown

<sup>5</sup>In-Process Controls

to curtail microbial growth to low levels should an excursion occur. This can only be shown empirically by performing HTS under the conditions specific to the process. Along with identifying potential points of microbial ingress, the HTS helps define the overall control strategy for both bioburden and endotoxin.

#### 8.1.2.10 Opportunities for Process Upgrade

If there is anything better than using biotechnology to cure disease, it may be by doing it faster, in greater volume, with a decrease in cost, and with increased safeguards. Given that biologics manufacturing is in many ways still in its infancy, there are ample avenues to be explored to increase safety, throughput and utility of biologics production. A single example will be given (a previous one mentioned was single-use bioreactors), that of changing the expression system to gain increased protein production in a shorter time with an expectation of reduced occurrence of impurities.

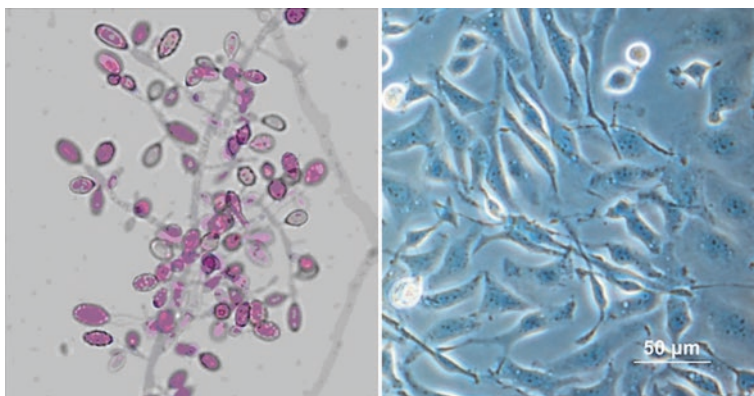
Emalfarb [61] relates efforts to improve the capacity of production by switching current CHO based expression to the use of a fungus called *Myceliophthora thermophila*.

...a drug platform utilizing the C1 fungal strain is already approaching 10 grams per liter in seven days, which is already far in excess of the industry average of approximately 4 grams per liter in 14 days. Because of the high productivity of the C1 fungal strain, an equivalent amount of drug may be able to be synthesized within a 2,000-liter bioreactor as is currently made using CHO with two 12,000-liter tanks. If so, the advantages of the C1 fungus could hold for capital expenditure and operating costs are clear.

The author also points out that not only much higher yields are possible, but also leaves less impurities to remove since there is “no risk of viral contamination” which thus removes two purification steps associated with traditional CHO purification. He says the C1 fungal strain has already received a “generally recognized as safe” designation from FDA. The C1 platform is produced using a proprietary strain of *Myceliophthora thermophila*, a similar strain is shown below (Fig. 8.11). Plant-based expression is also being explored and may produce some similar expression advantages [62].

#### 8.1.2.11 Cessation of Life-Saving Therapy

The reason for the existence of biologics is that they are life-saving drugs, thus it is important to state that any shortcomings are more than atoned for by their utility to patients. However, it also seems important to understand the historical discontinuity where it exists between modern biologics and historically antecedent drugs from a drug manufacturing and MCC perspective. Given the miraculous early history of some biologics, shocking successes that included rabies, smallpox and polio vaccines as well as early animal insulins, we should perhaps not be too



**Fig. 8.11** Conidia of *Myceliophthora thermophila* being produced from ampulliform swellings. *Lactofuchsin* stain, approximately 1000 $\times$ . (From Destino et al. [63]). At right, Chinese hamster ovary (CHO) cells typically used today in biomanufacture (public domain)

surprised at the miraculous advances that have been gained through the biotechnology revolution as each class of biologic presented above may include a dozen life-saving and quality of life-enhancing molecules that treat a wide range of afflictions. The lame walk, the blind see, the infected are healed, the healthy are shielded from future disease, malignancies fall away, those that are genetically lacking are made whole. This is the full fruit of the biotechnology revolution that continues to amaze. The downside of the “*life-saving*” or “*life-changing*” designations are the unfortunate few that must stop therapy due to adverse reactions. The improvement of a specific drug’s adverse event profile therefore has both a human and business component, in that attempts to reduce adverse reactions will help patients continue therapy as well as help drug manufacturers present fewer side effects in a competitive marketplace.

This is the flip-side of gaining the power to cure disease or, at least, vastly increase the utility of therapy: *the potential for denial of such treatment*. As a rather worst-case example of the consequence of terminating biologics therapy due to adverse events such as the development of antibodies against an administered recombinant protein an example will be given. Pure red cell aplasia (PRCA), which is “*caused by neutralizing antibodies to epoetin that cross-react with natural erythropoietin, produces a rapid decline in hemoglobin concentration, severe anemia, low reticulocyte count, and an almost total absence of red cell precursors*” (Baldo, 2017). This is an extremely adverse case referred to as “*breaking tolerance to self*”, where the formation of natural antibodies against a recombinant protein subsequently comes to imperil the continued existence of the body’s natural protein as it is also attacked (after all recombinant means identical). In such instances, the discontinuation of a life-saving drug, needed in this case for end-stage renal disease patients, is catastrophic.

Chapter 7 discussed the formation of antibodies against INF $\beta$ : “Neutralizing ADA (NAb) develop in up to 47% of patients using IFN $\beta$ -1b and up to 28% and 6% for those treated with s.c. IFN $\beta$ -1a and i.m. IFN $\beta$ -1a, respectively.” [64]

There have been attempts to circumvent the lack of tolerance to some recombinant molecules that share the natural amino acid sequence for patients developing neutralizing drug antibodies (NDAs) by putting the receptor on another protein scaffold with no amino acid sequence homology to the natural protein. However, in the case of erythropoietin, the use of a nonrelated peptide mimetic caused some patients to experience a different set of adverse responses, including anaphylaxis. This necessitated the voluntary removal of the new (replacement) drug from the market after several patient deaths. FDA acquired vials of the pulled medicine and associated the responses with the presence of subvisible particles in the manufactured vials relative to the vials used in the clinic for trials and upon which approval was based as described in Chap. 2 [65]. Though very rare, patients can also succumb to anti-drug antibody and other drug-associated side effects [66, 67].

### 8.1.3 Monoclonal Antibodies

Some basic immunological knowledge is necessary to follow developing trends specific to biologics microbiological control. Some immunological background was established in Chap. 7 that focused on a “simple model”. Without some basic immunological knowledge, we cannot appreciate the on-going sea-change in which the current “*endotoxin as pyrogen-only*” model of endotoxin control as being supplanted by control that includes added immune context.

#### 8.1.3.1 Antibody Basics

The immunological precepts below are not specific to monoclonal antibodies but antibody generation in general and, therefore, apply to natural (polyclonal) or therapeutic (monoclonal) antibodies.

- natural antibodies are produced only by plasma cells which are themselves derived from activated B lymphocytes
- the paratope or complementary-determining region (CDR) of antibodies that bind antigen are the amino acid sequences on the ends of the antibody Fabs (Y) produced (with the hope or anticipation of fitting) an antigen epitope
- the epitope consists of the amino acid sequences on microbial antigens that antibody paratopes bind to on the antigen
- one B cell/plasma cell produces one antibody of a specific paratope type and the secretion of the soluble antibodies from those cells match the membrane bound antibodies that act as receptors for the B cell itself

- there are millions of different CDR/paratopes that are the result of somatic (non-germ line encoded) rearrangements of the [v(d)j] genes that *are* germ-line encoded. In theory gene rearrangement could produce up to  $10^{11}$  different Antibodies [68]
- since the somatic rearrangements of paratopes are not germ-line encoded and are intended to detect structures on microbes that may not have even been developed yet (next year's influenza virus surface protein, for example), then they are called “*anticipatory*” structures and contrast the germ-line encoded (non-clonal) receptors (such as Toll-like receptors) that fit precisely to conserved microbial structures. The TLR4 complex (TLR4/MD-2/CD14) detection of endotoxin is an example of such a non-clonal receptor.
- the Fc (effector) end of the antibody binds to specific cell types to fulfil the dictates of the dual Fab (Y) antigen binding structures (for example, an Fc receptor provides a handle for phagocytosis of an antibody attached to complement at the Fab end).

The difference between mAbs and naturally generated antibodies is that a monoclonal antibody contains a singular paratope-binding sequence that has been cloned and cultured into many identical copies that bind a specific target epitope. If we consider a common protein such as insulin that can be antigenic if it derives from a mammal other than man (i.e. animal insulin), it is a protein that has only 51 amino acids but that has been shown to contain over 150 potential antigenic epitopes [69].

The antibody's 50 or so variable amino acids in its binding region define many overlapping groups of 15–20 amino acids. Thus, an antibody may fit many potential paratopes (such as shown in Figure 8.16 a and b). A paratope does not define a single complementary epitope; rather it presents certain molecular characteristics that bind antigenic sites with varying affinity [70].

An antibody “binding cleft” (Fig. 8.12) shows an antigen squeezed between the two arms of a single Fab.

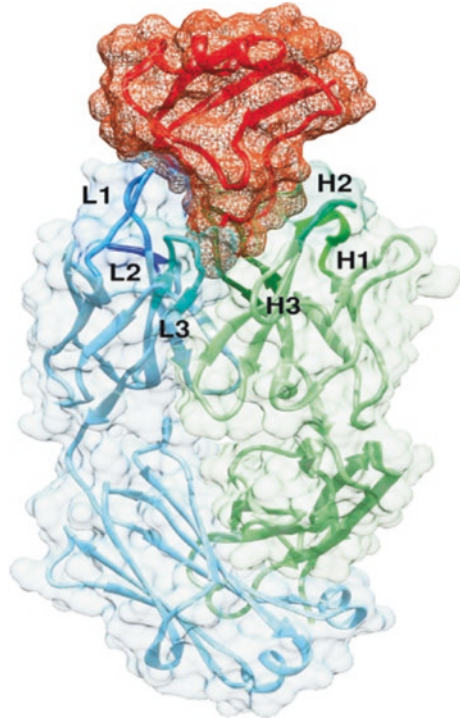
### 8.1.3.2 Nomenclature

The nomenclature of the scientific naming of mAbs has been systematically designated such that one can view a molecule's attributes simply from its name. Suffixes include humanized (zumab), human (umab) or chimeric (ximab) designations (Table 8.2 and Fig. 8.13).

### 8.1.3.3 Antibody Structure

Templeton and Moehle called the antibody structure a “dimer of dimers, two heavy (H) chains and two light (L) chains, with antigen recognition capability lying in the variable ‘head’ regions of an H-L pair.” The overall structure of a mAb is given by the IgG scaffold as shown below. Antigen binding sites are determined by

**Fig. 8.12** The fitting of a scorpion toxin (red) into Fab4C1 makes contacts with all six CDR loops of the H chain (green) and the L chain (blue). The authors [71] have described this as “an egg inserted small-end first in the egg cup”. The basket around the toxin represents the molecular surface of the protein in mesh style, and the surface around Fab antibody is rendered as a transparent solid. From Templeton and Moehle [72]



**Table 8.2** Nomenclature for monoclonal antibodies

INN substem (prestem) <sup>a</sup>		Disease Target <sup>b</sup>	
-o-	Mouse	-ba/b/bac-	bacterial
-a-	Rat	-ci/c-	cardiovascular/circulatory
-axo-	Rat-mouse chimera	-fuf-	antifungal
-e-	Hamster	-ki/k-	interleukin
-i-	Primate	-le/les-	inflammatory lesion
-xi-	Chimeric	-li/l-	immunomodulatory
-xizu-	Chimeric-humanized	-ne/n-	nervous system
-zu-	Humanized	-so/os/s-	bone
-u-	Fully humanized/human	-tox/toxa-	toxin
		-tu/t <sup>d</sup>	tumor
		-vi/v-	viral

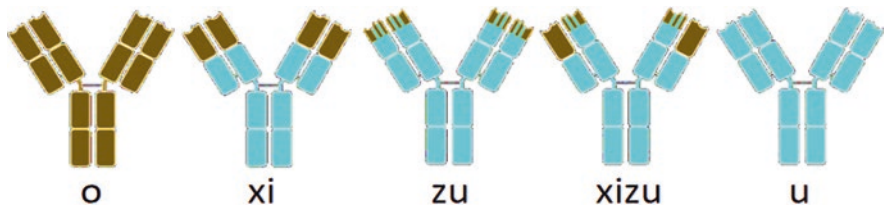
Derived from Baldo and International Nonproprietary Names (INN) for biological and biotechnological substances, 2016, World Health Organization

Example: **Palivizumab**. *Pali*, unique prefix identifier, *-vi-*, targeted to a virus (RSV); *-zu-*, INN substem for humanized; *-mab*, stem for all monoclonal antibodies

INN International Nonproprietary Name

<sup>a</sup>Further substem subdivision for tumors: *co/col* colon, *go/got* testis, *go/gov* ovary *ma/mar* mammary, *me/mel* melanoma, *pr/pro* prostate, *tu/tum* miscellaneous tumor

<sup>b</sup>All monoclonal antibodies have the stem (suffix) – *mab* and a unique prefix with no special meaning used to identify the individual product



**Fig. 8.13** Mouse sequences shown as dark and humanized sequences as light. Left to right, mouse antibody (O), chimeric (xi), humanized (zu), chimeric humanized (xizu), and (100%) human (u)

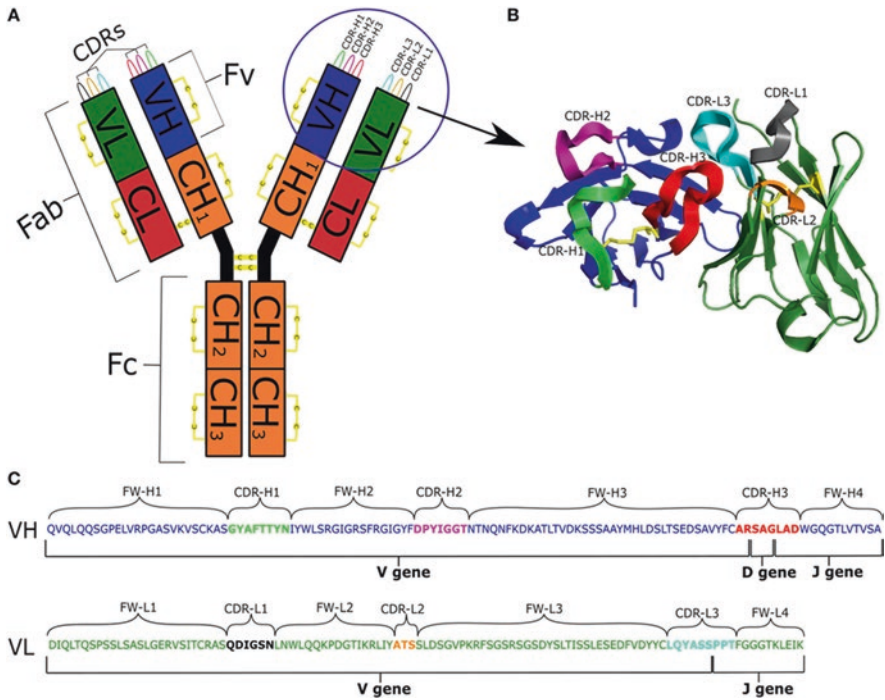
specific amino acid sequences on the variable light ( $V_L$ ) and variable heavy ( $V_H$ ) chains whereas the constant (C) regions are not subject to somatic V(D)J gene rearrangement. It is interesting that even in the variable regions, conserved or constant sequences between the variable segments [called the framework residues (FR)] make up about 85% of the  $V_H$  and  $V_L$  chains. This is an example of nature “*harnessing chaos*” to accomplish something that a set (hard-coded) structure (like TLRs) could not. The so-called *FRs* define the positioning of the paratope (CDRs) which is important to maintaining the overall structure of the antibody binding capability. If the extreme variability were allowed to “take over” the entire molecule, then it wouldn’t maintain itself as an “antibody”. The joining of the light and heavy chains forms a space between them on each arm of the antibody that is called an antibody binding “*cleft*” that is the antibody binding site (see Figs. 8.12 and 8.14).

Given this constant structural scaffold, the utility and specificity of monoclonal antibodies comes from a small set of six (three on each arm of the antibody Fab in the cleft) regions containing the fraction of highly rearrangeable (hypervariable) sequences. The number of amino acids per region is small (8–20) and thus, typically only includes perhaps 20–100 amino acid residues.<sup>6</sup> Figure 8.15 shows the many disulfide bridges (yellow), formed by the cysteine residues, that serves to hold the massive protein structure into one coherent functional unit of the characteristic shape (Y). It also shows the CDRs (paratope) as a function of both (C) the linear genetically produced amino acid sequence (bottom), (A) the common box diagram of antibody structure, as well as (B) the 3-dimensional folded form of the protein with CDRs which do not typically sit linearly next to one another. Figure 8.16 below that shows some areas of heterogeneity associated with monoclonal antibody production.

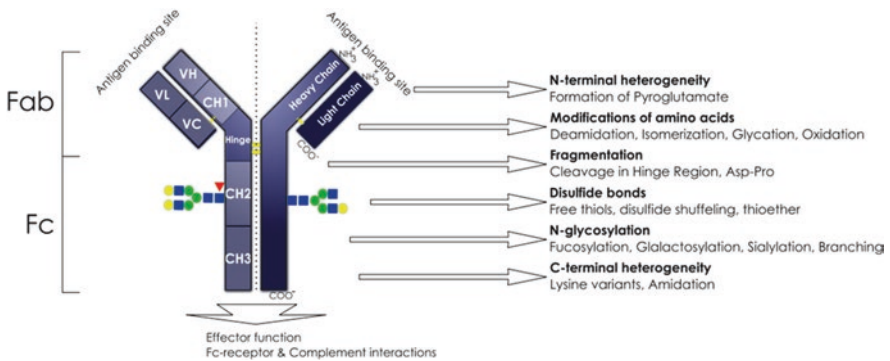
More specifically, as individual drugs, the mode of action of mAbs is to bind antigens and receptors for which they have been made. This can be seen below (Fig. 8.16a, b) in some detail for four different anti-EGFR mAbs.

<sup>6</sup>Different methods of determining the CDR sequences can give different estimations.

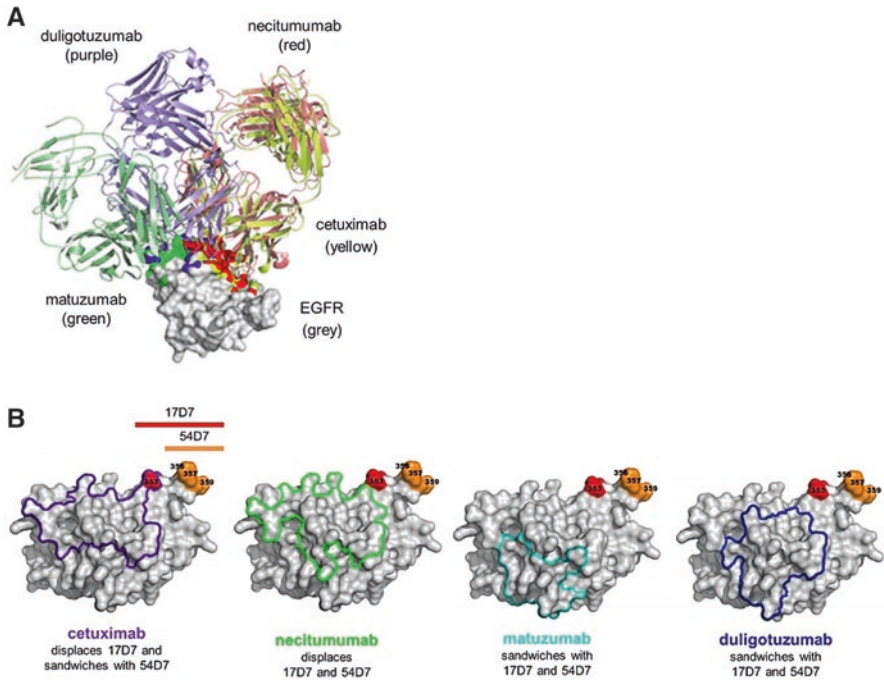




**Fig. 8.14** (a) Schematic representation of an antibody IgG structure. (b) Structure of the Fv region. (c) Genetic composition of VH and VL chains [IMGT numbering [73]]: VH is colored blue; VL is green; CDRs are labeled and depicted in different colors; and disulfide bonds are in yellow [74]



**Fig. 8.15** Schematic structure of an IgG1 molecule. An IgG antibody consists of two heavy and two light chains that contain several domains. The variable domains variable light (VL) and variable heavy (VH) that form the antigen binding site and the constant domains CL (constant light) and CH1–3 (constant heavy) form the structural frame. The IgG can be further divided into the Fab (fragment antigen binding) and Fc (fragment crystallizable) which induce the effector functions. (Shown on the right are possible modifications and alterations of the IgG frame [75])



**Fig. 8.16** (a) Kinetic and epitope characterization of four literature anti-EGFR mAbs. (a) Image of Fab:EGFR complexes when superposed via their EGFR domain III. EGFR domain III is shown in surface representation (white) while the different Fab fragments are shown in ribbon representation. Atoms that constitute the epitope for each Fab are colored in yellow (cetuximab), green (matuzumab), purple (duligotuzumab), and red (necitumumab). Buried surface areas were calculated as described under Materials and Methods. (b) Surface representation of EGFR (shown in grey) with an outline of residues that are buried upon complex formation with each mAb. This representation differs from that shown in A in that the outline comprises all residues that have change in surface burial, as opposed to showing only individual atoms that change burial. Thus A is a higher resolution representation of the buried epitope and B can be considered a lower resolution epitope representation. The residues identified from mutagenesis-based epitope mapping for mAb 17D7 (red) and mAb 54D7 (orange) are shown. Residues buried upon each Fab binding are shown in as an outline. (A and B are composite of Figs. 6B and 7D from Abdiche et al. respectively [76])

## 8.2 Part II Endotoxin Test Concerns of Biologics



### 8.2.1 Microbiological Risk Assessment

Endotoxin, of course, is a microbial by-product. It only comes from bioburden in a process or as an impurity in products manufactured in *E. coli* (or other GNB). Roche-Genentech developed a microbiological risk assessment process (Case by Case Assessment of Bioburden Excursions, CCAB) for biologics drug manufacture [77]. The risk of microbial contamination is real for biologics production. Since endotoxin is not the only PAMP associated with contamination, risk assessment is done using a variety of rationales for a number of different PAMP types of concern that includes also exotoxins. This was not a concern with SMDs that were typically chemically synthesized.

...the risk of microbial contamination is not limited to the final product. Many biologics manufacturing steps (e.g. protein purification, conditioning, formulation) occur under non-sterile conditions in aqueous systems at ambient temperature or 2–8 °C under substantially neutral pH conditions, making the large-scale production of biologics susceptible to microbial contamination.

The investigation of microbial excursions must adequately assess impact to both product quality and patient safety prior to making decisions regarding lot release. Development of a successful microbial control strategy for large-scale production of biologics should be based on a risk assessment to ensure end-to-end microbial control of the process through adequate prevention and detection. The risk assessments require review of the microbial quality of direct materials, utilities (WFI, process gases, etc.), and individual steps within the series of unit operations that constitute the DS and DP manufacturing process. For each step, the assessment should evaluate process/operational conditions and existing controls regarding the equipment, the manufacturing environment, and personnel preventive controls for their effectiveness at controlling and minimizing microbial contamination, with a goal of prevention of ingress, and proliferation in the process, equipment, and product. In addition, the existing microbial detection controls should be assessed to ensure they provide continued verification that the microbial prevention controls are working as intended. Detection controls consist of bioburden and endotoxin testing against established limits on samples obtained from defined process steps. For critical process steps (e.g. production culture and DS) the acceptable bioburden limit is extremely low and valid bioburden results exceeding acceptance criteria would lead to rejection of the batch.

The active pharmaceutical ingredient in biologics products are usually heat sensitive. Therefore, filtration using membranes with pore sizes ranging from 0.02 to 0.2  $\mu\text{m}$  size is the method of choice for bioburden reduction and terminal sterilization. While 0.02 to 0.2  $\mu\text{m}$  filtration can effectively remove intact microbial cells from the product, this process does not allow removal of subcellular structures of bacteria and fungi. If co-purified with the product subcellular structures like microbial toxins and so-called Pathogen Associated Molecular Pattern (PAMPs), i.e. endotoxins, lipopeptides, lipoproteins, flagellin, bacterial and fungal DNA, and cell wall polysaccharides potentially lead to toxic, allergic, or inflammatory responses in humans. In addition, co-purified extracellular proteases or endoglycosidases potentially lead to product degradation or modification. In other words, bioburden contaminations of non-sterile process intermediates represent a risk even after 0.02 to 0.2  $\mu\text{m}$  filtration, and even if both Drug Substance and Drug Product specifications are met. (von Wintzingerode)

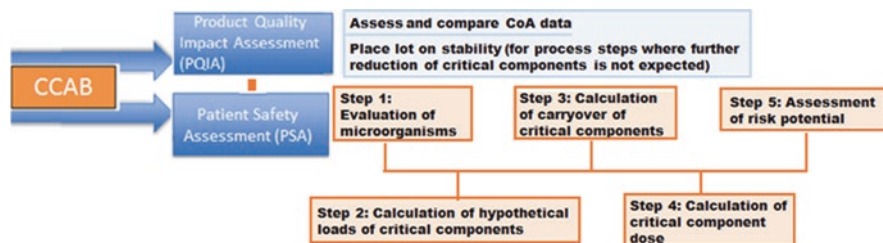


Fig. 8.17 Key aspects of Case by Case Assessment of Bioburden Excursions (CCAB) for biologics

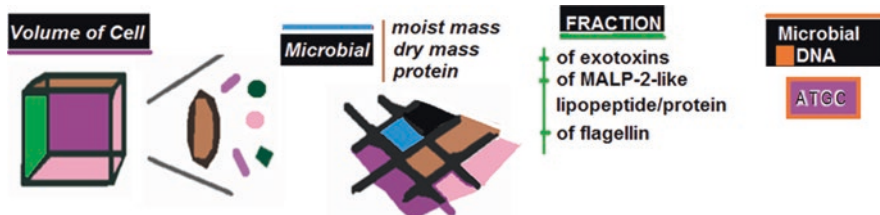


Fig. 8.18 Methods used to determine the theoretical extent of various contaminant types. For details around the calculation methods see immediate text below

The key aspects of the CCAB can be seen in Fig. 8.17 and the broad methods of estimating contaminant levels in Fig. 8.18. The more specific PAMP contaminant calculations levels are given as “worst-case” examples in Appendix I.

The following methodology was used by von Wintzingerode [78] to determine “worst case” potential contaminant per various cell types for biologics. Remember, since biologics are grown up in defined media rather than being solely water-based manufacturing as combined with synthetic API (SMDs), then non-Gram negatives are also of concern in preclusion methods. Methods used to determine the possible extent of various contaminant types is given below.

Based on published data and Roche/Genentech internal proteomic studies the hypothetical amount of critical microbiological byproduct per drug dosage were calculated as follows.

- Volume of cell<sup>7</sup> (1) Rods/ irregular rods (2) Cocci; different shapes use different formulas: (1)  $V_{\text{Cylinder}} = [\text{Max cell radius}]^2 \times \pi \times [\text{Max cell length}]$ , (2)  $V_{\text{Sphere}} = [\text{Max cell radius}]^3 \times \pi \times 4/3$ . See Müller and Löffler, 1992.
- Total Microbial moist mass (TMMM): “Moist mass of single cell” x cells/mL (CFU/mL); 1 CFU = 1 cell.
- Total Microbial dry mass: 30% of TMDM in contaminated sample, Schlegel, 1985
- Microbial protein: 55% of contaminated sample, Stouthamer, 1973

<sup>7</sup>Note: For molds the volume of single spores are calculated.

- Fraction of exotoxins: 0.05% of contaminated sample (Roche/Genentech internal study)
- Fraction of MALP-2- like lipopeptide/protein: 0.05% of contaminated sample
- Fraction of flagellin: 0.45% of contaminated sample (Roche/Genentech internal study)
- Microbial DNA: 4% of contaminated sample, Schlegel, 1985;
- Total cell wall content: 20% of *contaminated sample*. For fungi lower values (approx. 0.5%) are reported. Gottlieb & Van Etten, 1966.
- Fraction of cell wall polysaccharides:
  - Gram-negative bacteria: 10% of “*total cell wall content in contaminated sample*”, Schegel, 1985.
  - Gram-positive bacteria and fungi: 80% of “*total cell wall content in contaminated sample*”, Bartnicki- Garcia, 1968.

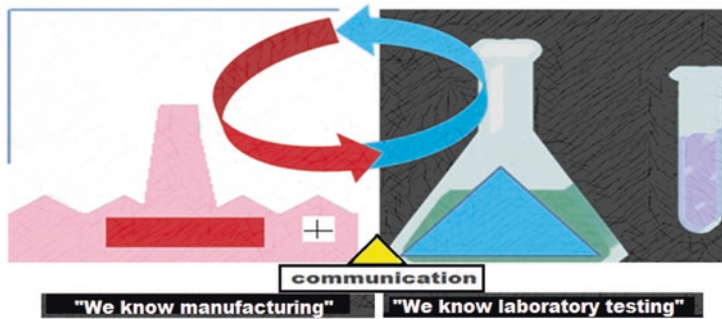
See Appendix I for specific recommendations based on worst-case calculations of known potent PAMPs and toxins. A second source of information for biologics risk assessment/control strategy comes from the European Biopharmaceutical Enterprises (EBE) concept paper on control of raw materials. Annex 1, Table 1 lists relevant (non-exhaustive) regulatory guidance for raw material QC. This is shown as Appendix II.

## 8.2.2 Protein-Endotoxin Binding

Endotoxin test laboratories should be aware of the potential for hidden endotoxin in protein solutions given a rather long history of its occurrence, even given expert methods of removal. Erridge [79] has put together a very good summary of this history from more of an immunological perspective rather than a microbiological perspective that includes both TLR2 and TLR4 PAMPs:

The possibility that experimental reagents may be contaminated with bacterial endotoxin (LPS) has been a consideration for researchers of immune mediators for decades [80, 81]. Indeed, the perceived immunological properties of several molecules have been found subsequently to be a result of endotoxin contamination [81–84]. Most frequently, this has been a result of the use of recombinant proteins expressed in *E. coli* [80, 81, 85–89], as used by 15 of the 64 studies summarized in Table 1 (not shown). In view of these concerns, however, most studies of potential endogenous TLR ligands have used alternative preparative techniques. For example, 26 studies used commercially sourced reagents or gifts from other researchers, 12 used reagents prepared from primary tissues (including blood), and five examined recombinant proteins expressed in mammalian systems. Thus, the established view is that most of the studies listed in Table 1 are likely to have used reagents with little opportunity for microbial contamination.

...recent evidence suggests that some caution may be required in the interpretation of the results of experiments using these control methodologies. For example, it is well established that the LAL assay is easily confounded by the presence of molecules that binds LPS. Indeed, LBP and bactericidal/permeability-inducing protein, were each shown to completely prevent the ability of the LAL assay to detect LPS [90]. Thus, it is possible that the LPS content of many of the proposed ligands could have been greatly underestimated if these ligands have any capacity to bind LPS. Moreover, the LAL assay is not able to detect lipopeptide



**Fig. 8.19** A knowledge gap may form between manufacturing that has the knowledge of complex removal methods and the laboratory that has the knowledge of *Limulus*-based test methods. The potential gap is the knowledge of the availability or binding status of endotoxin, including the isoelectric point of the protein in samples taken and sent to the lab for testing

contaminants [91], a notable consideration when almost half (28) of the reports suggest that their proposed agents stimulate TLR2 signaling. (Erridge)

### 8.2.2.1 Epistemology

Epistemology is the study of how we know what we think we know. From Merriam Webster: [92] *the study or a theory of the nature and grounds of knowledge especially with reference to its limits and validity*. Namely, how do we know that a protein solution is free of endotoxin when a protein may bind and thus mask endotoxin in a *Limulus*-based test? We can only know it by (a) releasing the bound pair and measuring it or (b) by understanding and precluding the conditions that bring such binding. The concern of this section is exemplified in Fig. 8.19 where each participant group is an expert in their domain, however, a knowledge gap can form between the two domains and impede communication and therefore operational performance.

The circular argument of whether endotoxin in a process can be seen or remains hidden is a problem of detection. We want to assume, after applying the *Limulus*-based tests that we have at our disposal, that the lack of detection equals the lack of occurrence of endotoxin. Yet, a study by Petsch et al. [99] demonstrates that this is not always the case, particularly with cationic proteins. They tried numerous methods *unsuccessfully* in trying to recover a spike of natural endotoxin filtrate into a range of biologic drug product solutions including:

- Phenol extraction
- Dilution with heat
- Perchloric acid treatment
- Trypsin digestion
- Chymotrypsin digestion
- Pronase digestion

The initial protease digestion method, the best of the bunch, only recovered 10–20% of the spike. However, what ended up working was the use of *proteinase K*



digestion with recovery up to 100%. Only after this treatment (breaking up the protein and dislodging the endotoxin) could LAL detect the added spike amount. In such cases, endotoxin-protein binding can “cause serious problems when cationic pharmaproteins are assayed for endotoxins: in these cases the LAL assay is not appropriate to determine the absolute amount of residual endotoxins, i.e., the biological risk of the product.” (Petsch et al.)

There are only a few laboratory methods available to ensure that proteins are not bound by endotoxin for test purposes, that is, in providing the needed orthogonal check of a protein solution prior to *Limulus*-based testing. It must be emphasized that routine testing of a process solution cannot always detect protein-bound endotoxin without some alternative method of treatment showing that the specific process solution in question does not contain protein-bound endotoxin. Some methods with the potential to do this are listed below:

- (a) use of a proteinase K as per Petsch et al.
- (b) a commercially available protease in kit form is from BDTI [93]
- (c) demasking via a method developed by Hyglos (acquired by bioMérieux) [94]
- (d) use of a cationic buffer -MgSO<sub>4</sub>, 1 M Tris buffer [95]

Each method comes with caveats. The caveat associated with the use of proteases, such as proteinase K that Petsch had used (described below) is that they often come with endotoxin bound to them, as they are cationic proteins that had to be purified too. Petsch et al. in their study admit that they subtracted off some small amount of endotoxin from the Proteinase K that was inherent in the material (as received). Chromatography is used in a serial fashion to remove all impurities and “polish” proteins to free them from minute amounts of impurities and contaminants including endotoxin. However, experts in the field are aware of the difficulty of removing endotoxin from cationic proteins. What may be less widely known is the masking effect of protein on *Limulus*-based detection methods.

Given the assumption that endotoxin may find obvious (as in when the expression system is *E. coli*) or subtle (bioburden residue) ingress into a process, the question arises: *by what mechanisms can such ingress remain “hidden”?* There are three potential avenues to be further explored including: (i) protein bound, (ii) low endotoxin recovery (LER) or surfactant masked, and (iii) endotoxin as an IIRMI (already discussed in Chap. 7). The tenacity of endotoxin-protein binding has been recognized (but only half heartedly embraced analytically) for many years. The historical proclivity for vaccines to be contaminated with endotoxin has been well documented. Similarly, Schwarz et al. found that many modern recombinant proteins (reagent not therapeutic drugs) routinely contain endotoxin at levels 2–4 times over the certified levels (also described in Chap. 7) [96].

### 8.2.2.2 Petsch et al. Study/Isoelectric Point

The Petsch et al. study [97] performed some 20 years ago perhaps best demonstrates the complex relationship of protein and endotoxin. They showed a large amount of endotoxin-protein binding at 1 mg/mL and pH = 7, conditions that may be considered



**Table 8.3** Summarized from Petsch, Deckwer, and Anspach's Tables 1 and 4

Cationic proteins	Isoelectric point, pI	EU/mL initial (LAL)	Endo + protein (LAL)		EU/mL post treatment - proteinase K (LAL)
			EU/mL	%	
Lysozyme	11.2	6180	297	<5%	6012
Rnase A	9.4	726	146	~20%	712
Human IgG	8.5-9.0	6180	99	<2%	5496 (~89%)
bFGF	9.6	478	9	<2%	Not shown
Anionic proteins					
BSA	4.7	6180	6100		NA- anionic
Murine IgG1	5.5	6180	5840		NA- anionic

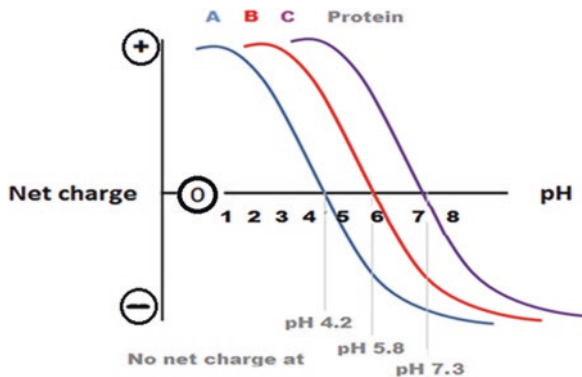
Endotoxin-protein masking and subsequent (proteinase K) demasking results. LAL test comparison of four cationic proteins (top 4) versus two anionic proteins (bottom 2). Notes: h-IgG is the molecular scaffold for mAbs and showed very high binding. There was no polysorbate or citrate in these solutions. Anionic proteins (BSA and mouse IgG1) presented no difficulty in detecting added endotoxin by LAL. Very large amounts of endotoxin (~ 6000 EU) were bound by the proteins. The endotoxin added was a natural endotoxin filtrate, not a highly purified standard.

much less concentrated and thus less strenuous than many current drug manufacturing processes [98]. Indeed, many biologic drug product concentrations are greater than 1 mg/mL, as 40–100 mg/mL are common [99]. With greater protein concentration comes greater potential endotoxin binding capacity. The pH is important because it determines the protein's charge and related activity relative to its isoelectric point (pI). The pI is the pH at which the charge of the protein is neutral (non-binding in solution). Petsch et al. study findings are summarized below in Table 8.3.

Human IgG is an antibody, and the basic scaffold upon which mAbs are based, and the binding of 98% of natural endotoxin (*E. coli* filtrate) such that the *Limulus*-based test result was <2% and, given that endotoxin can be released via a protease, exclusion of bound endotoxin is important. The blood stream is full of various proteases with a range of specificities [100]. It should also be pointed out that (i) the endotoxin loss above for the cationic proteins was achieved without a hold time period, (ii) used a non-purified endotoxin as spike and (iii) is a relatively minimal protein concentration (1 mg/mL) compared to many modern protein solutions manufactured. Today LER hold time spike studies are performed over a number of days. Therefore, without treatment one can safely assume that cationic proteins are not going to recover endotoxin via *Limulus*-based testing without some kind of pre-treatment.

Anspach summarizes the situation with removing endotoxins from protein solutions via affinity sorbents.

(Given) ...the extremely low endotoxin concentration in presence of substances up to 6 orders of magnitudes higher concentration, at which, owing to the high toxicity of endotoxins, still further purification may be necessary. Several consequences derive from these circumstances. The so-called negative chromatographic mode is preferred, which allows binding of a byproduct or pollutant—here endotoxins—whereas the product passes the adsorber without considerable retention. Elution of endotoxin is not the object, and therefore irreversible adsorption is an option; it may often be welcome. This is deduced from the fact that adsorption is an equilibrium process that, due the low endotoxin threshold levels



**Fig. 8.20** Manipulation of the pH of the drug sample milieu may allow for the release of bound endotoxin. Different therapeutic proteins can have very different isoelectric points (pI) as seen in Fig. 8.29 (mAbs). The pH at which the charge is neutralized is the pI. As shown, at a pH below the pI, proteins carry a net positive charge; above their pI they carry a net negative charge

expected, requires a very low apparent dissociation constant,  $K_d$ , of endotoxin and sorbent. This condition also follows, owing to unavoidable interactions with substances proteins of much higher concentration, leading to competition for endotoxin. The latter leads to the unfortunate situation of the endotoxin often being carried piggyback through an adsorber, the extent being dependent on the different strengths of interactions of the many components involved [101].

It is evident that some proteins are more difficult to purify than others. Those that, at neutral pH, are cationic (positively charged) stick strongly to negatively charged endotoxin. This binding with abundant protein may mask the presence of endotoxin when viewed via *Limulus*-based tests. In looking at human IgG, the basic scaffold for mAbs, the difficulty of removal can be seen in that even after protease (proteinase K) digestion (a drastic treatment that cannot be used except by destroying the protein endotoxin is bound to), over 10% of the endotoxin remains hidden (Petsch et al.). While chromatographic removal methods today are powerful, users should be aware that simple, straight-forward *Limulus*-based testing cannot verify the removal of endotoxin from cationic proteins without some orthogonal verification that it is has been removed, as Petsch et al. had showed.

It is important when sending a sample to the lab if labeled generically (e.g. “process run 6, sample 3”) to instead provide the needed context to help the test lab understand that they may be testing samples containing a cationic protein (Fig. 8.20). This is needed during process development and is not done post process development (as the process has then been validated and this is the process that will be used repeatedly). Manufacturing development should place a high priority on communicating that the endotoxin content determination cannot be truly realized without a check of the endotoxin *availability* in the matrix of the protein sample. This is true for both protein binding and LER phenomenon.

### 8.2.2.3 Pharma Manufacturing Views on Immunogenicity

Three main theories have come to explain the cause(s) of biotherapeutic immunogenicity. Chapter 7 discussed expert opinions on the surprise nature of immune responses to biologics therapy:

Despite the general trend towards ‘Humanization’, these drugs remain immunogenic in clinical settings, baffling drug developers. In principle, humanized and fully human monoclonal antibodies are ‘self’ immunoglobulins and should be tolerated. [102]

The three current prevailing paradigms can be characterized as:

- (i) **Mimetic-based**, where physical parameters associated with the product including protein aggregates, (visible and subvisible) particulates, and molecule heterogeneity (glycosylation, pegylation, etc.). This paradigm has already become deeply ingrained in biologics manufacturing operations.
- (ii) **IIRMI-based**, the impurity/contaminant-centric view (discussed in Chap. 7) is a sister to the mimetic view as both are based on adjuvanticity.
- (iii) **Epitope-based**, includes humanization and efforts to preclude pathogen mimicking epitope structures in therapeutic drug molecule development.

Manufacturing, which includes QC, personnel should have an appreciation for the various efforts that continue to go into reducing immunogenicity in biologics. This section seeks to highlight the role that manufacturing plays in the preclusion of artifacts (microbial and mimetic) that may contribute to immunogenicity. From the downstream vantage there is little widespread shared knowledge of the efforts upstream. Molecule development efforts have been exhaustive in attempts to, first “humanize” molecules and more recently to improve the prediction and preclusion of amino acid sequences that could be mistaken by the immune system as pathogen(-like) epitopes.

Adjuvant Effects: Mimetic and IIRMIN

Mimetic and IIRMI are both based upon the same adjuvant view, but consider that adjuvants used historically were not PAMPs like LPS but rather were (and still are) particulates, surfactants and emulsions. These have been used empirically for decades, even before the knowledge of the mechanisms of immune activation were known. Aluminum salts (alum), squalene, surfactants, and oil in water are still used in various combinations to provoke immune responses for various proteins [103]. The subvisible particle story in Chap. 2 demonstrates the slow uptake of adjuvant-based views in the pharma setting. From Chap. 2: Cherney summarized the immunological concern that subvisible particles present, including aggregated proteins, in a Feb. 2011 Pharma quality newsletter [104], explaining that for SVPs,<sup>8</sup> “the types and amounts of protein aggregates that may induce an immune response for our products are not clear,” Cherney provided recommendations on what firms should do in assessing and reducing risk in the face of this uncertainty. “I think one

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<sup>8</sup>SVP is subvisible particle

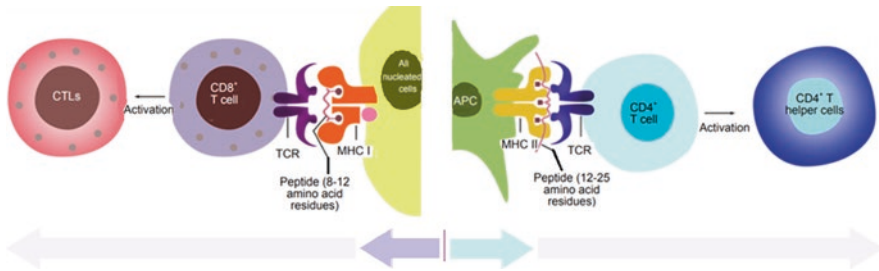
of the things you do,” he affirmed, “is that you document exactly how certain and uncertain you are about your product, and one of the ways to do that is to do risk assessment.” Today, with the hindsight of recent history, there is a certainty surrounding the importance of mimetic control in biologics processes, in controlling protein product aggregates, emulsions and surfactant interface interactions with proteins (even the silicone on a vial or stopper), as well as protein molecule heterogeneity (i.e. glycosylation, which also affects aggregation). The IIRMI addition to the established mimetic adjuvant view has already been thoroughly discussed in Chap. 7, but remains much less well disseminated from an industry perspective.

### Epitope-Based View

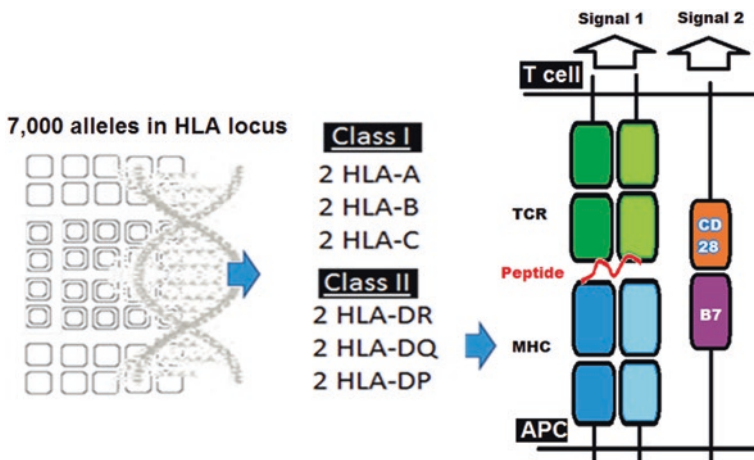
Antigen processing cells (APCs) have a specific way of processing proteins (including therapeutic proteins). They phagocytize large structures (large proteins, bacterial cells, virus particles, etc.) degrade them into short constituent peptides, and move these peptides to the surface for display on MHC I or II molecules where they are accessed by T-cells. The human proteins of which MHC consist are highly variable (human leukocyte antigen or HLA). Thus MHC present “samplings” of the immune milieu in a “right sized” format to TCRs as short peptides of 9 (8 to 11) or 20 (12 to 25) amino acids long for TCRs. It seems odd that such a short peptide could communicate what is “*friend or foe*”, after all, the proteins of every cell (human or pathogen) are made up of long strands of amino acids and breaking them down into very short random pieces seems intuitively non-productive. Yet, this is the method by which they are recognized as “self” or “foreign” to T cell receptors as matched with MHC molecules and as confirmed by additional signaling (such as CD28/B7). See Figs. 8.21 and 8.22. See Cousens, Moise and DeGroot for a generalized figure of the phagocytosis of a therapeutic protein ending in presentation of epitopes to T cells [105].

The idea that some small pathogen amino acid sequences of this size may serve as “*universal epitopes*” is explored here in an effort to understand (simplistically) the very complex topic of biologic molecule development. An example immunogenic epitope sequence from a pathogen is represented by the tetanus toxin (TTD) protein, which, for many years, has been believed to contain specific “*universal epitopes*” for human CD4<sup>+</sup> (MCH II) cells [106]. The tetanus toxin, from *Clostridium tetani*, is a devastating disease borne by the spore-forming anaerobes. The bacteria inhabit the soil and water and may contaminate wounds, in such an anaerobic environment (such as a puncture from a nail) they can proliferate and release toxins. Even though vaccination against tetanus has existed for decades, in 2013 it still killed 58,000 people worldwide [107]. A 1992 paper [108] lists TTD “*universal epitopes*” as: **TTD 830–843** and **TTD 947–967**. These specific epitopes have been referred to as “P2” and “P30” as they have been repeatedly used in historical vaccine research [109].

James et al. [110] (2007) disputed the idea of a “*universal epitope*” due to the presence of so many different MHC alleles in the human genome (7000 according to Sewell) [111]. This huge diversity is thought to be important to ensure survivors



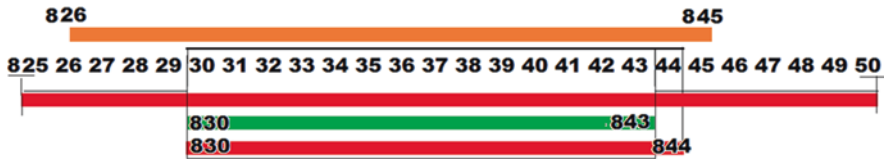
**Fig. 8.21** Differences in recognition and downstream processes between CD8<sup>+</sup> and CD4<sup>+</sup> T cells. CD8<sup>+</sup> T cells recognize pMHC-I complexes, where the peptide is a fragment from an endogenous protein typically 8 to 11 AAs in length, which occupies a groove that is closed on both ends. CD4<sup>+</sup> T cells recognize pMHC-II complexes, where the peptide is derived from cells or antigens engulfed by the APC and is typically longer, 12 to 25 AAs in length. The MHC-II groove is open on both ends. After activation, CD8<sup>+</sup> T cells differentiate into cytotoxic T lymphocytes (CTLs), whereas CD4<sup>+</sup> T cells differentiate e.g. into T helper cells, depending on receipt of further cytokine signals. APC: antigen-presenting cell; MHC: major histocompatibility complex; TCR: T cell receptor. (From: Tokuyasu et al. *J Cancer Metastasis Treat* 2018;4:2 DOI: 10.20517/2394-4722.2017.52. CC 4.0. Layout changed from vertical to horizontal orientation and arrows at bottom added)



**Fig. 8.22** The underlying diversity of TCR, MHC, epitope interactions. The TCR and MHC (I or II) have to pair based upon a myriad of inherited alleles. Class I and II representative alleles and allele class numbers are from Sewell. HLA is human leukocyte antigen, the gene complex encoding MHC proteins

after emerging infectious epidemics. By making clones of the various peptide producing tetanus toxoid sequences they found the following matches (TT to MHC):

...most of the TT-derived antigenic peptides were presented by 1 or 2 of the 10 alleles studied. However, there were some exceptions. For example, TT506–525, **TT826–845** (bold added) and TT1226–1245 were presented by three alleles; TT586–605, TT666–685 and



**Fig. 8.23** Overlapping sequences (epitopes) determined to be immunogenic (orange and red) or not to be immunogenic (green) in tetanus toxoid (TTD) amino acid sequences (short peptides or epitopes)

TT1234–1253 by four alleles and TT674–693 by six alleles. Because each of these peptides is 20 residues in length, it is possible that some may contain multiple epitopes while others contain a single epitope that is truly promiscuous.

These results contrast with the perceived notion that tetanus toxoid responses are dominated by universal CD4<sup>+</sup> T-cell epitopes. Rather these results illustrate heterogeneous T-cell responses for different class II alleles and individual-specific variation of the T-cell repertoire. (James et al.)

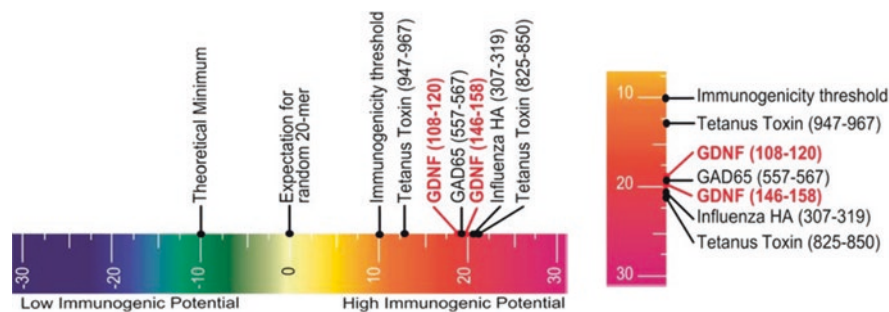
In other words, rather than being “*universal epitopes*” they were highly dependent upon the individual’s allele makeup and even a small sampling (10 of 7000) shows only a few matches. A 2014 paper lists TTD as **830–844**, which is largely in agreement with the first paper’s listed sequence [112]. However, although the most recent study using the most up-to-date methods (2017) [113] supported most of the historically defined “*universal antigen*” sequences, it refuted one of them, an important one, see Fig. 8.23:

Interestingly, nearly all of the previously described epitopes were contained within the predicted set, thus validating in an independent setting the results of the previous studies [114–117]. Conversely, no significant responses were detected against the “universal” TT epitope corresponding to residues **830–843** [118, 119]. (James et al.) (Bold added)

In the James et al. study the designated sequence overlap (**826–845; orange above**) does not represent the most immunogenic sequence (or the sequence with the most universal allele correspondence). It seems surprising that studies can differ on the immunogenicity of such a “*universal antigen*” structure as derived from a notoriously human pathogen toxin. Various sequences or subset peptides from TTD as shown above have been used in vaccines, particularly polysaccharide derived molecules, called conjugate vaccines [120], by attaching an immunogenic peptide (i.e. tetanus or diphtheria toxin epitopes) to the polysaccharide and thus generating an immune response against both moieties.

Weber et al. [123] provide an immunogenicity scale based on the “expectation for a random 20-mer” sequence [a random sequence of 20 amino acids, (“mer” from poly-mer)] that is assigned a “score” of zero. On this scale, shown below as Fig. 8.24, they have listed two immunogenic tetanus toxin sequences (108–120) and (825–850) which scored around 12.5 and 20 respectively and an influenza HA (hemagglutinin) sequence (307–319) that also scored around 20. Anything higher than 10 is “immunogenic”. The most immunogenic TTD sequence used employed a





**Fig. 8.24** This figure shows the EpiMatrix immunogenicity scale to prospectively predict clinical immunogenicity. It compares novel protein sequences to proteins of known varying immunogenic potential. The new protein therapeutic GDNF was about to be used as treatment for Parkinson’s disease or amyotrophic lateral sclerosis (ALS). In EpiMatrix analysis, however, it scores as high as published highly immunogenic peptides from influenza hemagglutinin and tetanus toxin. This might be the cause for the observed side effects during its clinical use as therapeutic. From Weber et al. [123]. Right side of graphic has been turned vertical to aid readability

sequence (TTD-**825–850**) that largely overlaps the sequence above that was found to be immunogenic historically but where a subset was found not to be immunogenic in the most recent study (**830–843**).

This short overview may exemplify some of the difficulty in definitively assigning epitopes as “immunogenic” or not.

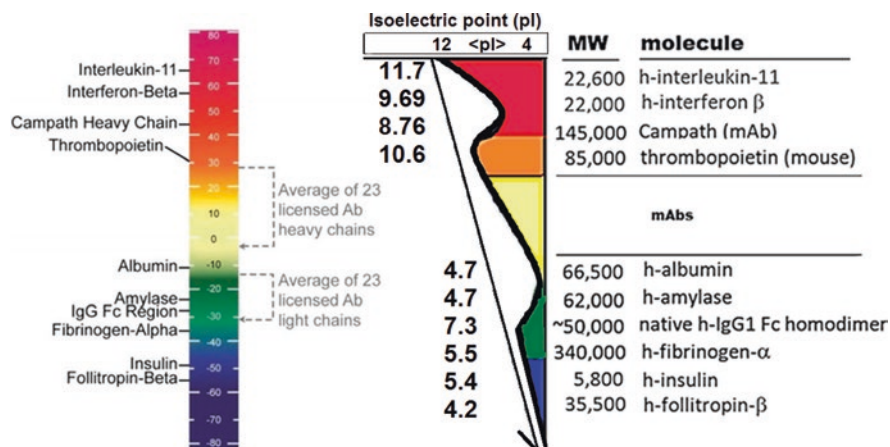
### Are the Three Views Relatable?

Since h-interferon- $\beta$  has been claimed as a cause of immunogenicity *by all three paradigms*: the epitope-based the mimetic-based [121] and IIRMI-based views (Chap. 7), it might be interesting from a manufacturing perspective to see if there is some relationship between the views. Here in Fig. 8.25 the adjuvant view (mimetic and IIRMI) will be combined into a single parameter: the isoelectric point (pI) of the protein, as Petsch et al. had used pI as a major dividing line of therapeutic proteins (basic, neutral, or acidic) that could be seen as correlating with the difficulty of impurity/contaminant removal processes as both (mimetic and IIRMI) are manufacturing-based rather than epitope-based perspectives. Figure 8.25 below shows a simple comparison of immunogenicity score by the epitope-based method relative to each protein’s isoelectric point.

This suggests that there may be some overlap in the two views, at least for the few proteins that have been given immunogenic “scale” values. Additional examples are shown in Table 8.4 below.

According to this scale the eight (four in Fig. 8.25 and four in Table 8.4) most immunogenic proteins (that range from 20 to 65 as compared to 12.5 and 20 for tetanus toxoid) are also the most cationic (and range from almost 8.25 to 11.7 in terms of isoelectric point) of the fifteen total molecules. Thus for this small, specific





**Fig. 8.25** Left: Summary of the immunogenicity scale findings for selected autologous proteins shows how antibody sequences rank, compared to standard controls. EpiMatrix protein immunogenicity scores higher than 20 are considered to be potentially immunogenic. Note that the low-scoring proteins on the lower left side of the scale are known to engender little to no immunogenicity while the higher scoring proteins on the upper left side of the scale are all known immunogens. For monoclonal antibodies, we adjust the antibody scores for the presence of pre-defined regulatory T cell epitopes [122] as we have evidence that the presence of these epitopes decreases the overall immunogenicity of antibodies in the clinic. Discovering these peptide sequences and identifying putative T cell epitopes may potentially be the most important aspect for protein therapeutic development. (From Weber et al. [123]). Right: added isoelectric points and molecular weights (Da) for molecules listed by Weber et al. (at left)

**Table 8.4** More molecules compared, pI versus immunogenicity based on epitope scoring

Molecule	Isoelectric point (pI)	EpiMatrix	
		color	~Score
GHRH (Growth hormone-releasing hormone)	10.54	Red	70
GM-CSF (Granulocyte-macrophage colony-stimulating factor)	5.2	Green	5
GDNF (Glial cell-derived neurotrophic factor)	9.23	Red	20
Infliximab	8.25	Red	26
Rituximab	8.68	Red	27

group of molecules, the most immunogenic molecules are also the most positively charged (cationic). These are all molecules of  $pI > 8$ . References for the  $pI$ 's cited are as follows: h-interleukin-11 [129], h-interferon  $\beta$  [130], Campath (mAb) [131, 132], thrombopoietin (mouse, predicted = 10.6) [133], h-albumin [134], h-amylase, (saliva 4.7–6.8 isoforms) [135], native h-IgG1 Fc homodimer [136], h-fibrinogen- $\alpha$ , 5.1–6.3 [137] h-insulin [138], and h-follitropin- $\beta$  [139].

The immunogenicity scale (Weber et al.) suggests that some proteins that are of human origin and that are produced as human recombinant molecules are much

more immunogenic than the tetanus toxin and influenza virus-derived sequences. A simple comparison as shown here (drug pI to immunogenicity) ignores many complex factors theorized to exacerbate immunogenicity. Any true, broad correlation would be surprising given the many powerful purification techniques that exist to overcome impurities. However, manufacturing processes and purification methods differ in the ability to remove impurities, company to company, process to process, molecule to molecule. There is no indication that any of the molecules above contain impurities as they have not been tested by methodology Haile et al. used (Chap. 7), where h-interferon- $\beta$  products were tested as the subject of the FDA study and which impurities (LPS and HCP) were present at levels satisfying legal limits.

Weber et al. (all expert immunologists) maintain that the proteins shown to be most immunogenic (Figs. 8.24 and 8.25 and Table 8.4) are immunogenic because they present sequences that resemble the short (12 to 25 amino acids) antigen epitopes (or multiple short sequences) that resemble those found in pathogens and that strongly bind MHC proteins thus increasing the effectiveness of presentation to T cell receptors. This has become the predominate way of viewing, and in some cases improving, biologic and vaccine protein therapeutics [140].

In keeping with the discussion of Chap. 7, Weber et al. describe the need for the co-stimulatory activation of T cells (via PAMPs), regardless of epitope sequence and regardless of the status as “*universal antigen*”.

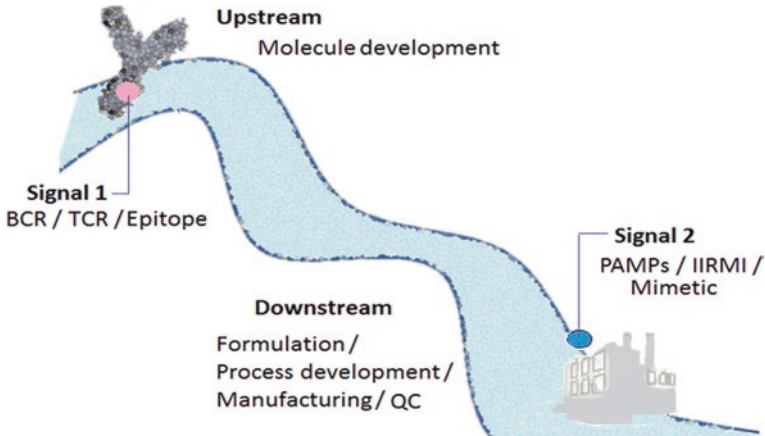
The controlling factor appears to be the “context” in which a T cell epitope is recognized by a T cell. If signals evoke release of inflammatory cytokines, resulting in expression of costimulatory factors by antigen-presenting cells, an effector immune response is triggered. In the absence of such signals, and the presence of others (such as an abundance of IL-2, or other regulatory cytokines such as TGF $\beta$ ), a T cell may be deviated towards a regulatory or tolerogenic response. Thus, for protein biologics, a T cell epitope may be friend (regulatory) or foe (effector) depending on the context of the response (Weber et al.)

TCRs are wildly diverse,  $\sim 10^{15}$  types per person per Sewell:

It is difficult to conceive of any obvious universal mechanism that might transmit knowledge of ‘presentable’ epitopes from previous infections between generations within the TCR CDR loops [141]. In the absence of ‘prior knowledge’ of the epitopes that might be encountered, T cell immunity must provide immune cover for all possible foreign peptides that contain appropriate anchors for binding to self MHC molecules. (Sewell)

This agrees with the assertion (Chap. 7) that it takes (at least) two separate sets of activities to create immunogenic proteins (as desired in vaccines but problematic in biologics), namely (i) the clonal receptor recognition of antigen (BCR/TCR) and (ii) the non-clonal receptor recognition of PAMP as second (co-stimulatory) signal (direct or as licensed to a non-PAMP receptor such as B7).

Thus the relationship between the two paradigms (epitope and adjuvant-based) could be viewed as a product of the mammalian immune system innate-adaptive immune response dichotomy: namely, the necessity of signals 1 and 2 respectively, where signal 1 is given by the lymphocyte receptor (BCR/TCR) and signal 2 is a co-stimulatory signal. Interestingly, this also describes the combination of upstream (molecule development) and downstream (manufacturing/QC) immunogenicity preclusion efforts (epitopes versus adjuvants including IIRMI and mimetics). See Fig. 8.26.



**Fig. 8.26** Molecule development and manufacturing as an extension of the signal 1 and signal 2 paradigm, spanning epitope and adjuvant-based (IIRMI, and mimetic) perspectives on immunogenicity causation

### Viral and Vaccine Protein pI's

According to recent literature, epitopes from pathogens are apparently not always recognized in a highly specific way. Sewell says “we should abandon the ‘one-clonotype–one-specificity’ paradigm suggested by clonal selection theory in favour of a ‘one-clonotype–millions-of-specificities’ reality.”

...the existence of extensive T cell cross reactivity means that heterologous immunity can extend beyond the cross-recognition of pathogens with high sequence similarity to allow, for example, BCG-induced T cells to also provide immunity against poxviruses [142]. Similarly, CD8<sup>+</sup> T cells specific for the human papillomavirus HLA-A2-restricted YMLDLQPET peptide also recognize the HLA-A2-restricted TMLDIQPED peptide from coronavirus [143]. Indeed, CD8<sup>+</sup> T cell mediated heterologous immunity can extend to very dissimilar antigens. For example, cells that are specific for the immunodominant GILGFVFTL peptide from influenza virus can often recognize the Epstein–Barr virus epitope GLCTLVAML [144] or the immunodominant HIV-derived SLYNTVATL antigen [145] (all of which are HLA-A2 restricted). (Sewell 2012).

As Sewell says, these later three pathogen epitopes seem not very similar as shown in Fig. 8.27.

There are other examples (besides that of therapeutic proteins) of disparate peptides (as epitopes) mimicking MHC/TCR binding and potentially contributing to autoimmune disease:

Molecular mimicry has been defined as similar structures shared by molecules encoded by dissimilar genes [146]. The molecular mimicry hypothesis postulates that T cells specific for microbial mimicking epitopes can cross-react with MHC restricted self-peptides. After an initial triggering and expansion of T cells by viral or bacterial peptide MHC complexes, the T cells can cross-react with self-epitopes in the periphery, potentially triggering autoimmune responses [147].

In this latter case, the viral protein does not share sequence homology with the human protein but rather shares conformational or 3D structural similarity: “The

Amino acid number:	1	2	3	4	5	6	7	8	9
influenza virus epitope	G	I	L	G	F	V	F	T	L
Epstein-Barr virus epitope	G	L	C	T	L	V	A	M	L
HIV-derived epitope	S	L	Y	N	T	V	A	T	L

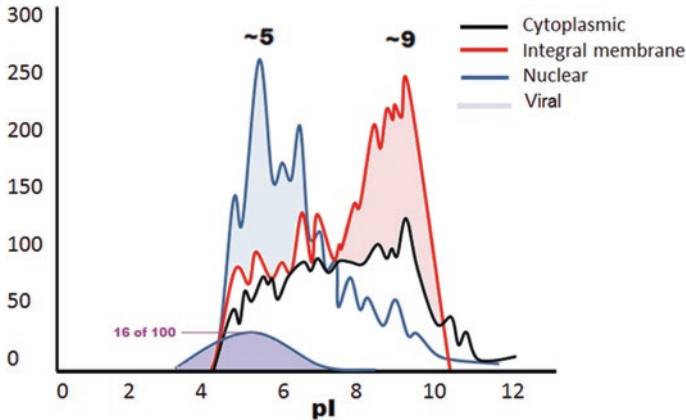
**Fig. 8.27** Lack of specificity of some overlapping MHC-binding pathogen epitopes according to Sewell

conformation of the rDBM and gp33 peptides is nearly identical despite a large sequence disparity.” (Sandalova et al.)

An interesting question can be raised in regard to recombinant vaccines that is essentially the reverse of the biologics question: *Is there some fundamental reason that vaccine subunit proteins from viral or microbial proteins would be less immunogenic than recombinant proteins from human sources?* Subunit vaccines have often not brought the needed immunogenicity desired as modern vaccines: “The high purity of these vaccines make adverse events less likely, but it also makes the vaccines less immunogenic and therefore potentially less effective.” [148] Therefore, the seeming contradiction arises: *Why are some human drug protein molecules immunogenic while many viral and microbial recombinant protein vaccine drugs are not?* After all, who is more likely to have epitope’s like pathogens than the pathogens themselves?

The immunogenicity potential for vaccines as administered drugs would not be a meaningful measure since every modern (subunit, recombinant) vaccine has an associated adjuvant and thus is inherently immunogenic (or else it wouldn’t be a vaccine). However, it is interesting to view the pI’s of various underlying vaccine proteins as produced without adjuvant, as microbial or viral proteins, to see, out of curiosity, where they fall as compared to human proteins. Some notes relevant to the pI of microbial and viral proteins versus eukaryotic proteins as purified constituents of vaccines are given below:

- Michen and Graule [149] determined the pI of 100 animal viruses to be within the range of 2.6–7.4 (excluding Encephalomyocarditis which was between 8 and 9).
- The polio virus as vaccine precursor described by Thomassen et al. [150] made from “complete intact polioviruses” has a pI of 6.2–7.4. Though the polio vaccine is not a subunit vaccine (it is inactivated as per Baldo), it shares a similar pI range as described above.
- Mehlin et al. [151] expressed 1,000 proteins from *Plasmodium falciparum* (the malaria parasite is a eukaryotic protist) in an *E. coli* expression system and the mean pI was 8.2. “The trend towards insolubility with increasing pI was striking: of the 288 targets with a pI above 10 only one was soluble. At very low pI values, there appeared to be a tendency towards a lack of expression which was compensated for by greater level of solubility among those which did express. The mean pI of these targets, including the tags, was 8.2.”
- Kiraga et al. [152] state that: “Schwartz et al. [153] and Knight et al. [154] found that membrane proteins are larger and more basic than non-membrane ones.”



**Fig. 8.28** “Integral membrane proteins and cytosolic proteins are found in large numbers in all proteomes. The two major protein clusters ( $\sim$ pI 5 and  $\sim$ pI 9) we have observed in the full proteomes of organisms belonging to the domains bacteria, archaea, and eukarya most likely corresponded to these two classes of proteins.” (Derived from Schwartz, Ting and King). Michen and Graule study of 100 viral protein pI’s added as purple curve (pI = 2.6–7.4)

Since most modern human therapeutic cell-surface-signaling proteins are membrane proteins, then these can be expected to have a higher pI as shown in a derivation of the Schwartz et al. figure above (Fig. 8.28).

Does Fig. 8.28 speak to the low immunogenic characteristics of many (subunit/recombinant) vaccine proteins as based on manufacturing considerations rather than epitope sequences? Most therapeutic human (non-vaccine) proteins being developed are CSSM’s which are therefore membrane proteins (shown above as red, pI 8–10). The pI of most viral and many microbial proteins (purple and blue) have lower pI’s and thus contrast many human proteins that are both larger (MW) and more basic (pI >8). The purified and expressed recombinant subunit vaccine proteins stand in stark contrast to historical highly immunogenic vaccines made of whole killed or attenuated (i.e. formaldehyde, see Chap. 2) organisms. This is clarified by Christensen:

Historically, vaccines have been based on live attenuated or killed bacteria and viruses i.e., smallpox, measles, polio and tuberculosis. These vaccines have in most cases proven very effective, as recognized by the eradication of smallpox and reduction in polio cases to below 100 cases of wild polio virus globally in 2015 ([155]). Although very effective, these vaccines often come with risks of side effects; such as fever, rashes, swelling and in some cases even vaccine derived infections, the latter being the case for one-third of all polio cases worldwide in 2015. Furthermore, large-scale production and ensuring consistency of the vaccine is very challenging, as demonstrated in a number of cases of global shortage of vaccines. One example is the decline in global availability of BCG vaccine against tuberculosis, where 180 million doses were required in 2015 to meet global demands, and only 107 million doses were made available from manufacturers ([156]).

To evade these issues, novel vaccine strategies have evolved where only the protective parts of the microbe are included, i.e. the so-called subunit vaccines. These can be produced in recombinant forms in yeast or bacteria yielding a product of high quantity, to be collected and purified. The currently registered hepatitis B vaccine is based on the hepatitis B surface antigen (HBsAg) produced in yeast cells. The high purity makes vaccine-induced adverse events

less likely, but it has also caused the vaccines to become much less immunogenic and thus less effective. There has therefore been a growing need for adding immune-potentiators, also known as vaccine adjuvants, to the vaccines to increase the immunogenicity. (Christensen)

Whereas the upstream development of molecules is a one-time per molecule endeavor (albeit a long and complex one), downstream manufacturing production and purification activities depend upon development and subsequent repeat performance activities lot to lot. Therefore, upstream, development scientists are charged with creating molecules that are less prone to immunogenicity and downstream scientists are charged with ensuring (i) that processes thoroughly remove impurities, (ii) PAMP signatures are certain to be detected and (iii) they cannot make overt or covert ingress into products. This latter is a young science from an immunology perspective given the only recent IIRMI discussion. The tendency is to think of humanization and epitope sequence makeup *as the singular paradigm governing immunogenicity preclusion efforts*. If so, it may serve to crowd out the adjuvant-based viewpoint and thus limit the potential for manufacturing development and performance advances.

Some care has been taken here to try and not draw too many conclusions from overly complex phenomenon, but to ask some questions based upon some interesting observations surrounding current complex theories of protein therapeutics manufacture and immunogenicity occurrence, which has improved for many molecules but persists stubbornly for some others, even some fully human proteins. The epitope-based view has quickly become deeply entrenched with successes extending broadly, from the use of toxoid epitopes in conjugate vaccines to the ultra-modern use of cancer (neo)antigen epitopes in T cell therapies [157]. The IIRMI-based view has only recently emerged but fits well with the, by now well-entrenched, mimetic view, as both share the adjuvant-based vantage.

#### 8.2.2.4 Process Development

The discussion of the importance of the isoelectric point in both process development and in subsequent lab verification of endotoxin removal studies (and likely as well as for other potential impurity and contaminant types) continues in this section. An assumption that masking may have occurred followed by some kind of demasking verification [158] should be an expectation to demonstrate endotoxin removal during process development, especially for cationic biologic proteins.

The isoelectric point of mAbs can differ substantially (~6 to ~9.5) despite the common IgG molecular scaffold. Figure 8.29 below from Goyon et al. demonstrates a range of isoelectric (pI) values [159]. However, it should be added that removal of endotoxin often employs Protein A as a first step and as such is not dependent upon the pI. The affinity of Protein A to mAbs occurs via attachment to the Ig Fc domain [160]. Goyon et al. discuss the method they used to determine pI and the importance of the pI value in monoclonal antibody purification.

cIEF<sup>9</sup> is a high resolution technique that separates species based on their pI or net charge, and takes into account solvent-exposed residues and internal amino acids. It has become the analytical platform of choice to characterize protein charge heterogeneity and determine

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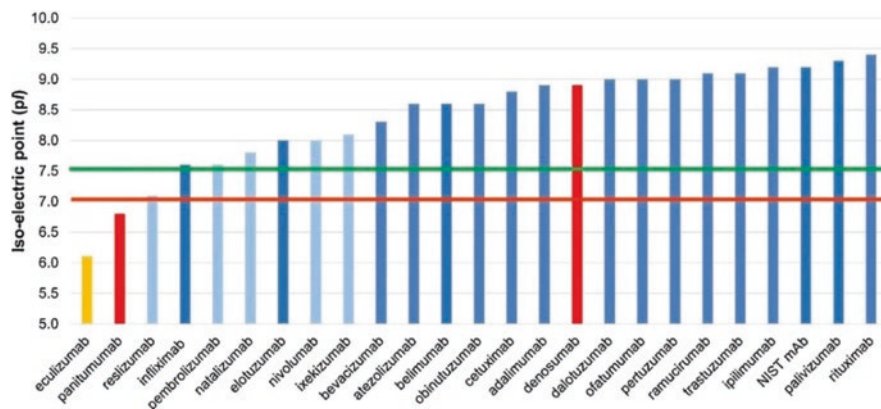
<sup>9</sup>cIEF is capillary isoelectric focusing



experimental  $pI$ s. This part aimed at evaluating the  $pI$ s of 23 FDA and EMA approved mAbs as well as dalotuzumab and NIST mAb by comparing experimental results and theoretical calculations. As shown in Fig. 1 (here Fig. 8.29),  $pI$ s measured by icIEF were comprised between 6.1 and 9.4. The coefficient of variations (CVs) for three replicates were lower than 0.1% for all mAbs, confirming the robustness of icIEF, as already demonstrated by an inter-laboratory study [161]. Typical  $pI$  values, comprised between 8.0 and 9.0, were obtained for 13 mAbs [162]. Except infliximab and elotuzumab, 15 IgG1 have a  $pI$  higher than 8.0, while most of the IgG4 mAbs have  $pI$ s lower than 8.0 (this feature was related to the higher amount of acidic amino acids such as aspartic acid and glutamic acid in the primary sequence of IgG4). Therefore, our findings confirm that most IgG4 mAbs are less basic than IgG1 mAbs, in agreement with the literature. The ionic properties of the mAbs are a crucial parameter for their purification, as most processes include at least one ion exchange step [163]. For antibodies with a  $pI < 7.5$ , anion exchange chromatography (AEX) can be used in flow-through mode to remove process-related impurities. CEX is subsequently used to remove charged process-related impurities for mAbs with a  $pI > 7.0$ . In this context, cationic mAbs ( $pI > 7.0$ ) [164] and especially those with a  $pI > 7.5$  may be selected during developability studies, to avoid specific purification processes (e.g. host cell proteins removal and viral inactivation steps [165]), therefore two  $pI$  thresholds limits were set at 7.0 and 7.5 in Fig. 1. Except reslizumab ( $pI = 7.1$ ), panitumumab ( $pI = 6.8$ ) and eculizumab ( $pI = 6.1$ ), 22 mAbs had  $pI$ s higher than 7.5.

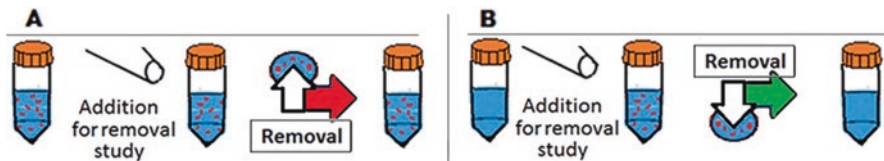
Therefore, in viewing Fig. 8.29 we can see that almost all mAbs at a pH of below 7.5 (horizontal green line shows 22 of 25) will have at least a small net positive charge. Furthermore, most mAbs at a pH of below 8.5 (15 of 25 shown above) will have a more positive charge. The positive charge binds negatively charged endotoxin.

Sometimes it is good to have generalizations: “Theory predicts that the solubility of a protein will be minimal near the  $pI$ ” (Shaw et al.) [166] Many proteins will precipitate out when pH equals their  $pI$ . For this reason the pH of natural blood proteins avoids the 7.4 spot (as do all of the mAbs shown above, as the closest are just above 7.5) and, interestingly, some have theorized that this  $pI/pH$  phenomenon plays a role in some diseases in which malformed proteins accumulate: “abnormal proteoforms in the blood”, including those “such as Alzheimer’s disease (AD),



**Fig. 8.29** Experimental  $pI$ s determined by icIEF for 25 mAbs (Data bars filled in yellow, red, light blue and dark blue for IgG2/4, IgG2, IgG4 and IgG1 types, respectively). (Goyon et al.)





**Fig. 8.30** Epistemology – the importance of a clean solution prior to endotoxin removal study. If an original solution contains bound (masked) endotoxin (a), then the removal of the added “load” (spike) does not appropriately demonstrate the removal of endotoxin. In fact, endotoxin bound (masked) prior to a reduction study will likely make removal of added endotoxin easier as existing endotoxin occupies or pre-saturates the binding spots. Hidden endotoxin should be shown not to occur (as Petsch et al. had done), then the study can proceed as per (b) knowing that it is free of potentially masked endotoxin

Amyotrophic lateral sclerosis, prion disease, Creutzfeldt-acob disease, Parkinson’s disease (PD), amyloidosis and a wide range of other disorders” [167].

The relationship of pI and protein binding can affect the performance of endotoxin removal studies. Such studies are used to demonstrate removal of endotoxin by forward processing, generally establishing a log reduction value (LRV), for specific unit processes. As *Limulus*-based testing is applied to samples received from manufacturing that are intended to show endotoxin reduction by various process studies, at various steps, then the pI and the pH of the process solutions should be taken into account to determine if protein binding is likely to affect the study. Figure 8.30 shows (A) the simplistic assumption that pI and pH are not relevant to such a study, thus the study may start with bound endotoxin and make removal look better than it is. Or (B) a solution that has been pre-screened for bound endotoxin and can be forward processed appropriately. Only method (B) will provide great confidence in the result for a cationic protein.

### 8.2.2.5 Resin Cleaning

The use and reuse of resins for impurity/contaminant removal shows deterioration over time and thus may add a concern regarding the consistency of endotoxin removal.

Chromatography resins and membranes are typically re-used multiple times during production scale downstream purification of biologics. A key consideration that necessitates their repeated use is the high cost of chromatography media. This is an especially important consideration during Protein A chromatography which has become the workhorse of monoclonal antibody and Fc fusion protein purification processes. A critical aspect is to ensure that the chromatography resin can continue to deliver product which meets predetermined quality and safety attributes over the lifetime of the resin.

Several mechanisms can contribute to a decay in the useful lifetime of a chromatography resin over repeated uses. Impurities and/or residual product can remain on the resin and either block pores and/or block access to surface ligands. An additional consideration for Protein A resins is that of leaching or degradation of the ligands over time, particularly as a result of harsh regeneration conditions. In general, the maximum number of reuses for any given chromatographic stationary phase is product specific and depends on a variety of factors including the resin used, the placement in the process, the level and nature of impurities

the resin comes in contact with, the product itself and the nature of the strip, regeneration and column storage solutions used [168].

Resins are expensive and come with built-in assumptions in regard to recommended depyrogenation procedures. This from Boulet-Audet, Bernadette Byrne and Sergei G. Kazarian: [169]

Cleaning-in-place (CIP) protocols typically include washing the resin with alkaline solutions [170–172] to prevent contaminant build-up. Sodium hydroxide solutions can efficiently remove precipitated proteins, lipids and nucleic acids while inactivating bacteria, viruses, yeast and endotoxins [173, 174]. High pH conditions during CIP also inactivate microbes while removing contaminants that could carry over into subsequent purification cycles [175]. However, even with CIP, the mAb binding capacity of the Protein A resin binding capacity decays over purification runs, typically requiring replacement after 50 to 300 cycles [170, 176]. Replacing a single industrial scale 1500-L protein A column can cost up to \$12 M, not including the incurred production interruptions [177]. Important cost savings could result from optimisation of CIP protocols to extend the resin lifespan.

Protecting the resins from microbial growth is a tension inherent in regenerating column resins and the removal of opportunities for microbial growth. Use of a higher concentration of NaOH for Protein A column cleaning is desirable since it improves the efficacy of column sanitization to prevent microbial growth during column storage, yet too high a concentration can damage the column. Since the regeneration condition is predominantly responsible for the decline in capacity, efforts are made to screen and identify additives to the solution that would protect the ligand while still allowing for effective resin cleaning and sanitization.

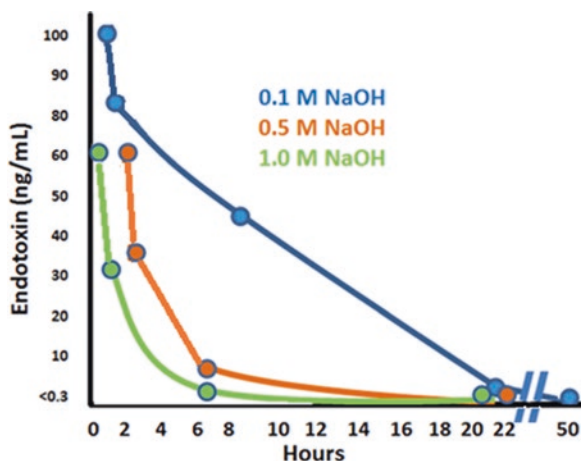
Large amounts of microorganisms such as yeast and bacteria can destroy the function of chromatography columns and media. These organisms can also have indirect effects, such as clogging of filters and other system components as well as produce harmful substances such as endotoxins, enterotoxins, and proteases. Table 2 (not shown) shows that sodium hydroxide is very effective in inactivating a number of yeasts and bacteria and that this inactivation is dependent of concentration, contact time, and temperature. It is clear from Table 2 that bacterial spores might not be totally inactivated by sodium hydroxide [178].

Table 2 mentioned above is not shown, because it shows bacterial not endotoxin reduction. Endotoxin is not as easily inactivated as are bacteria or even bacterial spores and these kinds of studies are highly empirical and thus need to be demonstrated for each specific setup. The mechanism of alkaline hydrolysis is believed to result from the saponification of fatty acids. In an old but classical study, Niwa et al. showed that hydrolysis in base (NaOH) without heat reduced *E. coli* LPS pyrogenicity, but only moderately without the application of heat or alternatively with addition of alcohol.

Alkaline degradation of endotoxin as ordinarily carried out in aqueous solution requires several hours at rather high temperatures and strong alkali to obtain appreciable detoxification and decrease in fatty acid ester content. For example, treatment with 0.25 N NaOH at 56°C for 2 hr resulted in significant detoxification, whereas 0.1 N alkali at 20°C for 24 hr was insufficient to effect complete detoxification... Treatment with sodium hydroxide for longer intervals does not affect their antibody-neutralizing and erythrocyte-modifying capacity but results in a marked loss of toxicity and pyrogenicity [179].

Figure 8.31 below shows the expected reduction of resin-resident-endotoxin using various NaOH treatments.

**Fig. 8.31** Inactivation of endotoxin by NaOH. (Adapted from GE Application Note [180])



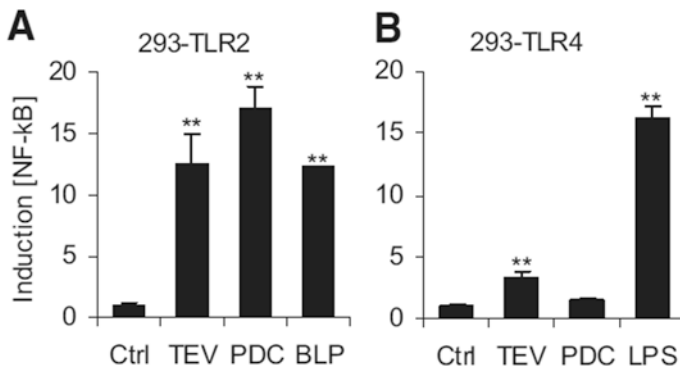
Historically, heat has been viewed as a necessary accomplice to alkylation depyrogenation efficacy. It should also be pointed out that NaOH treatment, while seemingly effective, raises some questions about alkyl-treated activity of “depyrogenated” LPS. This brings up the distinction between endotoxins that have been inactivated as gauged by *Limulus*-based testing and those that have been (only) detoxified and could remain active from an adjuvant-like perspective. The rather antiquated study referenced above by well-regarded endotoxin pioneer’s points to the contrasting concepts of adjuvanticity (immune stimulation) and pyrogenicity which today has been largely absent from discussion.

Erridge describes the potential for microbial impurity ingress via reagent proteins and eluates of protein purification columns (Fig. 8.32).

...recent evidence has revealed that endotoxin and lipopeptide contamination may be more prevalent in commercially sourced reagents than appreciated previously [81, 91]. For example, we have found that the eluates of commonly used protein purification columns can be contaminated with BLP,<sup>10</sup> despite the use of ethanol as a preservative in such columns (Fig. 1, shown here as Figure 8.33). Moreover, enzymes used routinely for protein tag removal in mammalian recombinant protein expression systems, such as TEV protease, or other preparative enzymes may be commonly contaminated with LPS and lipopeptide, presumably reflecting their recombinant origin (Fig. 1). A further concern regarding the examination of recombinant proteins derived from mammalian expression systems is that some surveys have suggested that as many as 25% of mammalian cell lines may harbor ongoing mycoplasma contamination [181]. Findings such as these suggest that significant potential for LPS or BLP contamination may exist for reagents used in any of the studies listed in Table 1, including those that have not used bacterial expression systems.

Vaccine research, in always searching for new adjuvants, has used NaOH for producing detoxified endotoxin, which can produce low-pyrogenic or muted proinflammatory responses yet retain the immune stimulating ability (adjuvanticity) as a cellular remnant of GNB.

<sup>10</sup>BLP is bacterial lipopeptide



**Fig. 8.32** Potential sources of contaminants in non-prokaryote recombinant systems. HEK-293 cells transfected with a NF-κB-sensitive reporter, CD14 and TLR2 or TLR4/MD2, were challenged with a 1:100 dilution of commonly used tag removal enzyme TEV protease, saline eluate from an unused protein-desalting column (PDC), 100 ng/ml Pam3CSK4 (BLP), or 10 ng/ml LPS. \*\*,  $P < 0.01$ , versus medium alone [control (Ctrl)]. (From Erridge et al.)

Like *Escherichia coli* LPS, the NaOH-induced VPGs<sup>11</sup> are an excellent activator of pro-inflammatory cytokines (IL-1 $\beta$  and iNOS), anti-inflammatory cytokine (IL-10), and dual activities (IL-6) in the stimulated macrophage cells.

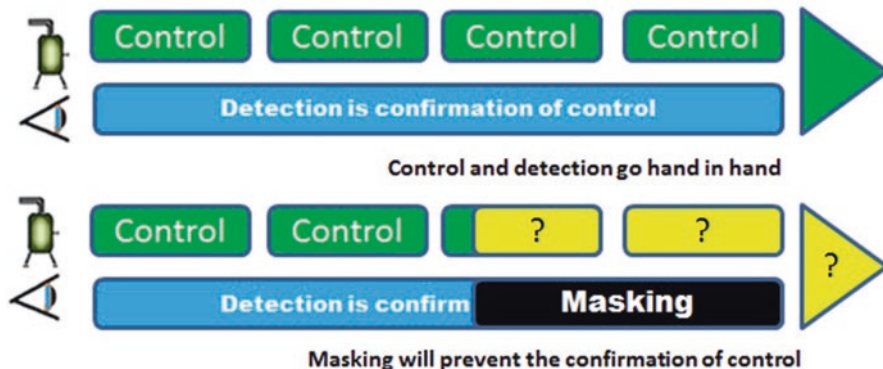
All these results indicate that NaOH-induced VPGs show the potency of a safe, economical, and effective inactivated vaccine candidate [182].

Microorganisms can grow in resin columns and these columns are treated with caustic (NaOH); subsequently, several potential concerns may arise. Has the treatment truly depyrogenated the resin from *both* pyrogen and adjuvant perspectives? Can remaining bacterial shells, or “ghosts” or LPS or other PAMP molecules remain as immune stimulating contaminants? Stated alternatively, can LPS detoxified in NaOH retain immune stimulating properties even if the eluates have been confirmed to be depyrogenated via *Limulus*-based testing? This recurring concern of biologics manufacturing processes arises only recently as deriving from a perspective that includes an immunological context (PAMPs as adjuvant co-stimulatory signals and not simply pyrogens).

### 8.2.3 LER HTS Performance/Demasking Overview

The Low Endotoxin Recovery (LER) issue first identified by Chen and Vinther [183] has upset the BET testing status quo. Some have differed philosophically with FDA whom has sought to address the lack of ability to recover spiked endotoxin from undiluted drug product that has served to dissociate (chelator) and mask endotoxin by abundant polysorbate molecules in specific formulation types (polysorbate

<sup>11</sup>“Acellular bacterial ghosts (BGs) are empty non-living bacterial cell envelopes, commonly generated by controlled expression of the cloned lysis gene E of bacteriophage PhiX174. In this study, *Vibrio parahaemolyticus* ghosts (VPGs).” Ibid.



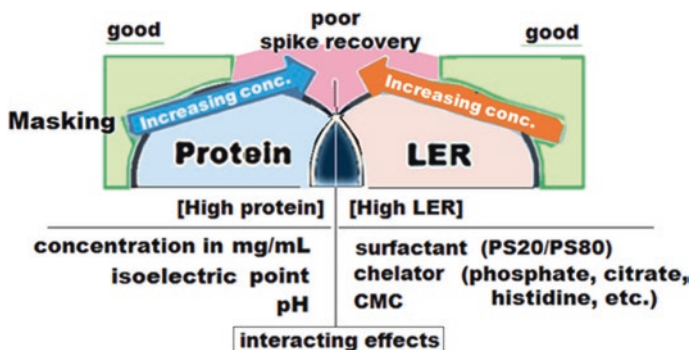
**Fig. 8.33** The loss of detection (blue) via masking (black) doesn't imperil endotoxin control, however, the loss of detection is the loss of the confirmation of control. Thus, masking brings into question continuing control since contamination is not prevented once and for ever by a control strategy but rather requires specific performance and detection within each unit process and in the production of each repeat drug batch

with chelator). Thus endotoxin cannot be detected in such a state and if such a state exists in downstream unit processes, then it could pose a potential risk of missing the detection of endotoxin. As discussed above the protein binding phenomenon (Petsch et al.) has existed for decades with little recognition that the phenomenon may affect specific drug testing, likely because it made little difference to LVP or SMDs (that typically do not modulate immune reactions).

Here is a good place to distinguish endotoxin control from detection. Control centers around the many elements associated with a control strategy (as discussed here and in Chap. 6) whereas detection is necessary to confirm control which is a QC quality expectation. The distinction between control and detection (as confirmation of control) functions can be seen below in Fig. 8.33.

Schwarz et al. [184] presented evidence that *Limulus*-based masking of formulated protein solutions via the LER phenomenon, though preventing the detection of endotoxin, nevertheless supports the expression of key cytokines when masked endotoxin is present including IL-6, IL-12, CXCL8 (chemokine interleukin 8) and TNF- $\alpha$ . Furthermore, LPS masking does not prevent the activation of immune cell surface molecules from such solutions. As we saw in Chap. 7, the activation of cell surface molecules is the means by which therapeutic molecules target and modulate disease processes.

Upon stimulation with LPS, monocytes up-regulate the activation markers and co-stimulatory molecules CD40, CD80 and CD83, whereas CD86 expression is down-regulated [185, 186]. These co-stimulatory molecules are especially important during the activation of T cells [187]. To investigate whether stimulation with masked LPS could also produce an increase in surface activation markers on monocytes, we analysed the surface expression of CD40, CD80, CD83 and CD86 by flow cytometry... Overall, our data show that masked LPS is a potent inducer of immune responses in primary human monocytes. (Schwarz et al.)



**Fig. 8.34** The potential for overlap in the protein and LER endotoxin masking phenomena. Factors affecting the severity of each is listed

CD80 and CD86 are important co-receptor participants in the co-stimulation of CD28, one of the strongest receptors governing T cell activation (and Chap. 7 gives an idea of how many biologics drugs now modulate this pathway). It's activation is confirmation of the immune modulating activity of masked LPS that need not be pyrogenic or active via *Limulus*-based testing to exert immune-stimulating effects.

In a biological drug milieu there may be several interacting phenomenon occurring with a given protein formulation that does not give good direct spike recovery. Two phenomenon include protein binding and LER. However, there is little discussion of the overlap of the two phenomena, since practically speaking, masking is masking. Depending on the specific drug there may be multiple opportunities for masking. See Fig. 8.34 below.

A PDA Task Force was formed in 2015 to further the understanding of the low endotoxin recovery (LER) phenomenon as well as to help standardize the performance of endotoxin hold time studies for LER (LHTS). Endotoxin LHTS are required by FDA to demonstrate that endotoxin spiked into undiluted product can be recovered prior to routine end-product QC testing. As a summary of the expectations of such studies, the following points should be considered (as elaborated in the PDA Technical Report 82 on LER (2019):

- The LHTS is required on 3 different lots of a final biologic drug product for submission in a Biologics License Application (BLA)
- A spike using CSE or RSE should be made into the original or suitable container of undiluted drug product (liquid or as reconstituted)
- The test method to be used should be the same as that to be used in the final QC test
- The spike level should be relevant to the specification and should target the mid-point of the standard curve for recovery purposes
- To minimize assay variability the study can be performed in reverse mode and carried out for at least 7 days
- The LHTS should mimic the conditions of manufacturing in terms of hold time and temperature of hold



- Recovery of 50% or greater must be achieved over the course of the study. If two consecutive time points are <50%, then, by definition, LER has occurred.
- FDA has stated that if LER cannot be overcome, then a company must release-test individual lots of biologic using the rabbit pyrogen test (RPT) *on an interim basis* until an *in vitro* de-masking test can be developed
- A BLA post-approval commitment will be required to be developed as an (non-rabbit) *in vitro* test to overcome LER for the specific product

While the LHTS is not technically challenging, overcoming LER in a specific protein product can be very challenging because each approach has to be tailored to the specific product formulation (protein with excipients). Although FDA is requiring a rabbit pyrogen test as an interim solution for LER masked solutions, longer term they are requiring the development of more appropriate tests (via BLA post approval commitments). The rabbit pyrogen test (while holding legal significance for biologics testing) is relatively insensitive and can only be used as a “*fever or no fever*” tool. RPT is not a sensitive test in comparison with *Limulus*-based tests which use common sensitivities of 0.01, 0.005 and 0.001 EU/mL.

Table 8.5 below shows a summary interpretation of PDA Technical Report 82 case studies 1 through 12. Note that complex studies are difficult to briefly summarize. Readers should consult original report for items of interest. See all PDA TR’s here: <https://www.pda.org/docs/default-source/website-document-library/bookstore/pda-technical-documents-list.pdf>.

**Table 8.5** Summary interpretation of PDA Technical Report

Case study subject under study and product constituents	Mitigation method(s) and/or other takeaway
<b>1</b> <b>PS content relative to protein content.</b> Effect(s) of freezing sample. Mab 0.006%, DS 120 mg/mL, DP 90 mg/mL	Protein in the absence of PS20 did not show LER. Increase in protein content showed greater LER effect using same PS %. <b>Storage at ultralow temps (<math>\leq -30</math> °C) showed improvement</b>
<b>2</b> <b>Evaluate storage conditions post spike (10 EU/mL CSE).</b> Biosimilar 50 mg/mL protein-citrate & PS20 via reverse HTS	<b>Risk assessment:</b> “...provides a means of defining risk categories and levels and suggests mitigation approaches for the varying risk levels.”
<b>3</b> <b>Study “... the susceptibility of crude naturally occurring endotoxins (NOEs) and purified endotoxins...”</b> Creation of synthetic LER solutions (PS 20/80) and citrate/phos. buffers. No protein	“As shown here in several case studies, that LER is a function of purified endotoxin cannot be concluded as the <b>majority of crude NOE preparations also suffered LER-based recovery loss.</b> ”
<b>4</b> <b>Temp., pH, and salt concentration.</b> Creation of synthetic LER solutions (PS20/80) & citrate/phos. buffers. No protein	<b>Effect of HTS dilution with MgCl<sub>2</sub>.</b> “...controlling the temperature of samples and using magnesium dilution method for hold-time studies is important.”

(continued)



**Table 8.5** (continued)

Case study subject under study and product constituents	Mitigation method(s) and/or other takeaway
<b>5</b> <b>Using different methods for calc. of % endo recovery from spiked products.</b> Synthetic LER solutions. No protein	"... knowing the starting amount of endotoxin is critical." <b>"Method II (using LPS recovered from the spiked LRW as the denominator) is superior to Method I and Method III....</b> PS spiked into LRW exhibited remarkable stability over the duration of the hold time in all four studies."
Method I used INITIAL Water Spike (WS) time point. Method II used CORRESPONDING WS time point. Method III used THEORETICAL WS	
<b>6</b> <b>LPS masking and resolution in Mab,</b> pH = 6.0, 25 mg/mL protein. 20 mM citrate, 150 mM NaCl, 20 μM DTPA, 0.025% PS80	<b>Use of cation replacement buffer (0.5 M MgSO<sub>4</sub>, 1 M Tris)</b> optimized for LAL, method, product formulation used (DS, DP). "... satisfactory hold time recovery studies of CSE were achieved by adding a sample pretreatment and dilution step using cation replacement buffer."
<b>7</b> <b>"Develop a sample preparation protocol in a problematic sample matrix."</b> Blinded protein sample with LER configuration. Protein content not specified	<b>Endo-RS® demasking (bioMerieux).</b> "the demasking procedure should be able to detect endotoxin from different species... To show broad applicability of the demasking protocol, the RSE and three different NOEs... were incubated in the sample and subsequently demasked."
<b>8</b> <b>Investigate and overcome LER driven by protein alone.</b> No LER constituents, 1 drug product, 2 solid drug substances	Reverse HTS design reduced variability. <b>Introduced "sample-specific hold times based on data and by implementing in-process controls for the DP and DSs."</b>
<b>9</b> <b>LER case study for an Mab.</b> High [protein] Mab with chelator (EDTA) and surfactant (PS80) and histidine	Only one LAL kit prevented LER for up to 14 days (>50% recovery). <b>In some specific formulations, simply switching LAL may aid HTS recovery.</b> Routine testing would require this test be used
<b>10</b> <b>CSE recovery for DS with LER constituents.</b> Citrate buffer and polysorbate 20 in DS; process bulk w/o LER solutions	"... implementing a <b>risk-based</b> , in-process control throughout the drug substance and drug product manufacturing process"
<b>11</b> <b>No LER effect in a sodium phosphate, PS80 formulation matrix,</b> 5 mg/mL final protein, 25 mM sodium phosphate, 0.0325% PS80, 13% mannitol buffer	"... <b>no LER effect was observed</b> (for this matrix)... as the recovery on T8 was still 59.8%." Sometimes LER is not observed with specific formulations that might be expected to show it
<b>12</b> <b>No LER in biologic with known LER-causing ingredients.</b> Protein content not specified, L-histidine, PS 20, Sodium dihydrogen PO <sub>4</sub>	<b>"...formulators can add (this formulation) to their choices</b> for when selecting non-LER-causing ingredients to formulate their unique biological products."

PS polysorbate, DS drug substance, DP drug product

<sup>a</sup>12 case studies from Parenteral Drug Association (PDA) Technical Report (TR) 82 (2019)

## Appendix I. Specific Recommendations Based on Worst-Case Calculations of Known Potent PAMPs and Toxins

Class of PAMP/Toxin	Worst case example and Rationale	Limit
Non-protein exotoxins	<b>Tetrodotoxin</b> , Clinical study of tetrodo-toxin - potent neurotoxin [188] (Hagen et al., 2011)	30,000,000 pg/patient
MALP-2 like Lipoprotein/protein	<b>Compared to TDLo of LPS</b> (literature search) Since bacterial lipopeptides can be shown to be endotoxic on account of their chemical structure (Schromm et al., 2007), they can be assumed to have a potency comparable to that of MALP-2 or LPS	4000 pg/kg body weight
Flagellin	<b>Vaccine-recombinant fusion protein incorporating flagellin</b> , (Turley et al., 2011). Fused flagellin shows an increased immunogenicity compared to native flagellin (Huleatt et al., 2007)	1,000,000 pg per patient
Protein exotoxins	<b>Botulinum toxin A (BotA) exotoxin</b> is the most potent toxin of all to humans (Alouf and Popoff, 2006). The reported toxic dose low (TDLo) is 0.14 units Dysport®/kg (Braune et al. 2001), 115 U Dysport® correspond to 1 ng of botulinum toxin A [189]	1.2 pg/kg body weight
Bacterial or fungal DNA	<b>SS DNA</b> , Clinical studies of Immuno- stimulatory DNA (SS) vaccines (Higgins et al., 2007). Very high doses of ISS (15 mg/kg) result in severe effects in mice. This value is more than 300-fold higher than ISS doses used in vaccine trials, which have shown a low toxicity of ISS (Higgins et al., 2007)	50,000 pg/kg body weight
Cell wall poly-saccharides	<b>GNB</b> - Toxic dose Low of LPS is 4 ng/kg <b>GPB</b> - LTA <b>Fungi</b> - $\beta$ -glucan Potency of LTA is at least 100× less potent than LPS. Peptidoglycan (PG) is at least 10,000× less potent than LPS (Kimbrell et al., 2008), (Rocel and Hartung 2012). Fungi: Toxicology based limit of $\beta$ -glucan (internal study)	Bacteria: 400,000 pg/kg body weight Fungi: 800,000 pg/kg body weight

Derived from von Wintzingerode (2017)

## Appendix II. Guidance Related to GMP and Quality of Raw Materials (Non-exhaustive)

References – Relevant content related to Raw Materials	
<b>ICH M4Q</b>	<ul style="list-style-type: none"> <li>• Quality section of the Common Technical Document: Culture media and other additives (details provided in 3.2.S.2.3)</li> <li>• The description should include information on, for example, scale, buffers and other reagents (details provided in 3.2.S.2.3), information on the quality and control, information demonstrating that materials (including biologically-sourced materials, e.g., media components, monoclonal antibodies, enzymes) meet standards appropriate for their intended use (including the clearance or control of adventitious agents) should be provided, as appropriate. For biologically-sourced materials, this can include information regarding the source, manufacture, and characterisation. (Details in 3.2.A.2 for both NCE and Biotech)</li> </ul>
<b>ICH Q3D</b>	Guideline for elemental impurities
<b>ICH Q5A (R1)</b>	<ul style="list-style-type: none"> <li>• Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin</li> <li>• It is recommended that manufacturers develop programs for the ongoing assessment of adventitious viruses in production batches. The scope, extent and frequency of virus testing on the unprocessed bulk should be determined by taking several points into consideration including the nature of the cell lines used to produce the desired products, the results and extent of virus tests performed during the qualification of the cell lines, the cultivation method, raw material sources and results of viral clearance studies.</li> </ul>
<b>ICH Q5D</b>	Guideline derivation and characterisation of cell substrates used for production of biotechnological/biological products
<b>ICH Q7</b>	<ul style="list-style-type: none"> <li>• Good manufacturing practice</li> <li>• No (raw) material should be released or used before the satisfactory completion of evaluation by the quality unit(s)...</li> <li>• The quality unit should establish a system to release or reject raw materials, intermediates, packaging and labeling materials</li> <li>• Specifications should be established and documented for raw materials..... Acceptance criteria should be established and documented for in-process controls</li> <li>• The (API) impurity profile should be compared....in order to detect changes to the API resulting from modifications in raw materials,.....</li> </ul>
<b>ICH Q9</b>	<ul style="list-style-type: none"> <li>• Quality risk management.</li> <li>• This guideline provides principles and examples of tools for quality risk management that can be applied to different aspects of pharmaceutical quality, including the use of raw materials.</li> <li>• To assess the critical attributes of raw materials, solvents, Active Pharmaceutical Ingredient (API) starting materials, APIs, excipients, or packaging materials</li> </ul>

References – Relevant content related to Raw Materials	
<b>ICH Q11</b>	<ul style="list-style-type: none"> <li>• Development and manufacture of Drug Substance</li> <li>• The manufacturing process development program should identify which material attributes (e.g., of raw materials, starting materials, reagents, solvents, process aids, intermediates) and process parameters should be controlled. Risk assessment can help identify the material attributes and process parameters with the potential for having an effect on drug substance CQAs. Those material attributes and process parameters that are found to be important to drug substance quality should be addressed by the control strategy</li> <li>• The quality of each raw material used in the manufacturing process should be appropriate for its intended use. Raw materials used in operations near the end of the manufacturing process have a greater potential to introduce impurities into the drug substance than raw materials used upstream. Therefore, manufacturers should evaluate whether the quality of such materials should be more tightly controlled than similar materials used upstream</li> </ul>
<b>9 CFR Part 113 sections 50, 52, 53</b>	<ul style="list-style-type: none"> <li>• Requirements for ingredients of animal origin used for production of biologics</li> <li>• 113.50 — Ingredients of biological products</li> <li>• 113.52 — Requirements for cell lines used for production of biologics</li> <li>• 113.53 — Requirements for ingredients of animal origin used for production of biologics</li> </ul>
<b>• 21 CFR 610.15, 21 CFR 211 Subpart E and 21 CFR 211.110</b>	<ul style="list-style-type: none"> <li>• 21 CFR 610.15: constituents shall meet generally accepted standards of purity and quality</li> <li>• 21 CFR 211 Subpart E: Control of components and drug product containers and closures; components are required to be controlled by a Quality Control to ensure appropriate management. Testing and monitoring of components...components should be tested for identity and for conformity for purity, strength and quality.</li> <li>• 21CFR 211.110: In-process materials shall be tested for ID, strength, quality and purity as appropriate, and approved or rejected by the quality control unit...</li> </ul>
<b>USP &lt;1043&gt;</b>	<ul style="list-style-type: none"> <li>• USP-NF General Chapter &lt;1043&gt; Ancillary materials for cell, gene and tissue-engineered products</li> </ul>
<b>USP &lt;1074&gt;</b>	<ul style="list-style-type: none"> <li>• USP-NF General Chapter &lt;1074&gt; Excipient Biological Safety Evaluation Guidelines</li> </ul>
<b>ChPh 2015</b>	<ul style="list-style-type: none"> <li>• Quality Control Procedures for Raw Materials and Excipients Used for Production of Biologics</li> </ul>
<b>Ph.Eur.5.2.12.</b>	<ul style="list-style-type: none"> <li>• Raw Materials of Biological Origin for the Production of Cell-Based And Gene Therapy Medicinal Products</li> </ul>
<b>Ph. Eur. Monograph 2034</b>	
<ul style="list-style-type: none"> <li>• Substances for Pharmaceutical Use</li> </ul>	
<b>EMA</b>	<ul style="list-style-type: none"> <li>• EMEA/CHMP/410869/2006 Guideline on Human Cell-Based Medicinal Products</li> </ul>
<b>EMA</b>	<ul style="list-style-type: none"> <li>• EMEA/CHMP/QWP/396951/2006 Guideline on excipients in the dossier for application for marketing authorisation of a medicinal product</li> </ul>

## References – Relevant content related to Raw Materials

**Directive 2009/120/EC**

- amending Directive 2001/83/EC of the European Parliament and of the Council on the Community code relating to medicinal products for human use as regards advanced therapy medicinal products

**Eudralex Volume 4, Annex II**

- Good Manufacturing Practice (GMP) guidelines

**EU guideline 2015/C 95/02**

- EU Guidelines on the formalised risk assessment for ascertaining the appropriate good manufacturing practice for excipients of medicinal products for human use

**Other**

- PDA Strategies for Controlling Raw Materials in Biologics Manufacturing
- by Annemarie Möritz, PhD, Novartis Pharma AG | Jan 05, 2015
- <https://www.pda.org/publications/pda-publications/pda-letter/latest-news/2015/01/05/strategies-for-controlling-raw-materials-in-biologics-manufacturing>

From EBE [190]

## Appendix III. Available Chromatography Methods

Method	Main features
Direct washing	with alkali ethanol, nitric acid and 70% ethanol in ultrasonic bath
Microfiltration & ultrafiltration Based on membrane absorbers.	Convective mass transport has shorter path length which translates to shorter residence time. Both filtrations show relatively good endotoxin clearance
Monolith	(i) PGMA has large pores which translate to rapid mass transfer, high adsorption kinetics and negligible low-flow-resistance (ii) Addition of EDTA provides for the regeneration of metal-chelated particles with no morphology damage or protein adsorption capacity loss
Particle based adsorbents	Endotoxin and protein adsorption using DMAPAA takes place under a low salt condition
Two phase micellar system	(i) involves separation of aqueous surfactant solution into micelle-rich and micelle-poor regions through excluded-volume interactions (ii) external agent notably Triton X-114 is needed to maintain the inherent biological activity of protein while reducing the endotoxin level by 100-fold
Affinity chromatography	(i) Poly( $\epsilon$ -lysine) derived cellulose beads provide greater endotoxin selectivity (ii) High endotoxin retention is promoted using small pore size based on size-exclusion effects Affinity chromatography (iii) The high selectivity eliminates the need for multiple purification steps and reduces production costs (iv) Reproducible, scalable and capable of specifically recognizing and purifying super-coiled pDNA in arginine affinity chromatography (v) Arginine being non-immunogenic avoids interference with endotoxin assays

Method	Main features
Immobilised metal affinity chromatography (IMAC)	(i) It can be applied for RNA, pDNA and endotoxin removal (ii) There are situations involving special affinity interaction such as pure plasmid DNA binding specifically only to Fe <sup>3+</sup> charged chelating compound
Size-exclusion chromatography (SEC) and ultrafiltration	(i) It uses composite polyacrylamide as the column which is highly porous (ii) Ultrafiltration is used if protein is not present. This method is capable of removing large endotoxin aggregate with alkanediol as one of the many agents used for effective endotoxin removal
Anion-exchange chromatography (AEC)	It has rapid separation, wide selection of AEC media, sodium hydroxide (NaOH) sanitisation and does not require any solvents
Cation-exchange chromatography (CEC)	(i) Is thought to be more efficient than anionic exchanger in terms of endotoxin removal (ii) Polycationic ligands offer extremely strong attraction/binding for endotoxins (iii) PEI as a hydrophilic polymer has superior biocompatibility and exhibits hydrophobic interactions with endotoxin while PLL works well for protein recovery and still usable after binding capacity exhaustion
Immobilized hydrophobic ligands & reverse-phase chromatography (RPC)	Interact with non-polar protein surfaces through van der Waals forces for high endotoxin removal

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