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The clearance of viruses and transmissible spongiform encephalopathy agents from biologicals

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The viral and transmissible spongiform encephalopathy (TSE) safety of therapeutics of biological origin (biologicals) is greatly influenced by the nature and degree of variability of the source material and by the mode of purification. Plasma-derived and recombinant DNA products currently have good viral safety records, but challenges remain. In general, large enveloped viruses are easier to remove from biologicals than small 'naked' viruses. Monoclonal antibodies and recombinant DNA biopharmaceuticals are derived from relatively homogeneous source materials and purified by multistep schemes that are robust and amenable to scientific analysis and engineering improvement. Viral clearance is more challenging for blood and cell products, as they are complex and labile. Source selection (e.g. country of origin, deferral for CJD risk factors) currently occupies the front line for ensuring that biologicals are free of TSE agents, but robust methods for their clearance from products are under development.

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Introduction

Product pathogen safety (i.e. keeping products free from contamination with pathogenic organisms) is especially important for therapeutic products of biological origin (biologicals) and is greatly influenced by the nature and variability of the source material and by the modes of subsequent purification. It has been especially difficult to assure that biologicals are free of viruses and the infectious agents causing transmissible spongiform encephalopathies (TSE agents or prions). Biologicals can be broadly divided into two categories: products containing, manufactured using or derived from human or animal tissues or fluids (e.g. plasma derivatives that are manufactured from highly variable source material); and biotechnology products derived from recombinant and transgenic sources with well-defined starting materials. Currently, both categories of product have good pathogen safety records. In the past, however, products derived from human tissues or fluids were implicated in the transmission of infectious pathogens. For example, blood derivatives such as clotting factors and immunoglobulins have transmitted viruses [1], and human growth hormone (hGH) extracted from the pituitary glands of cadavers transmitted Creutzfeldt–Jakob disease (CJD) to some recipients [2]. Thus, precautionary steps must be taken to ensure the virus and TSE safety of biologicals (Table 1). Risk reduction strategies for a given category of product depend on the origin of the starting materials and subsequent purification. However, strategies for assuring the safety of all biological products share common elements: careful source selection and testing for pathogens, if possible, and the inclusion of pathogen clearance steps in the manufacturing process, when feasible.

Source selection and testing are intended to minimize the risk of transmitting known infectious agents or, at least, to minimize amounts in the starting material. Screening and testing depend on the source and nature of the starting material. For example, donors of blood and plasma are selected by use of a screening questionnaire to identify risk factors for relevant infections, and their donations are tested for markers of infection with HIV, hepatitis B virus (HBV) and hepatitis C virus (HCV). For biological products prepared in cell cultures, origins of transfectants and hybridomas must be documented and cell banks themselves are tested for contamination.

The second precautionary step leverages the ability of the purification processes to clear infectious agents, either fortuitously (as by chromatography or precipitation) or deliberately through the introduction of dedicated viral-clearance steps into the process (e.g. by filtration and solvent/detergent). The pathogen clearance capacity of purification steps can be estimated by conducting validation studies (Figure 1). Virus clearance studies have often been performed for manufacturers by specialty contract laboratories. Preparations of model viruses are spiked into an input feedstock (the starting material, often prepared from actual manufacturing intermediates), and this

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Table 1

Typical safety strategy for a recombinant DNA or monoclonal antibody biopharmaceutical[a].

Strategy level	Test/procedure	Added safety factor
Cell bank screening	Sterility	Eliminates cell lines contaminated with bacteria and fungi
	Mycoplasma detection	Eliminates cell lines contaminated with mycoplasma
	In vitro adventitious virus screen	Eliminates cell lines contaminated with viruses that can grow in the substrate
	In vivo virus screen	Eliminates cell lines contaminated with inapparent viruses and some Rickettsia
	Antibody production assay monitoring antivirus antibody production in mice or hamsters injected with a sample from the cell bank (e.g. MAP, HAP)[b]	Eliminates cell lines contaminated with a defined panel of viruses
	TEM	Identifies cell lines with virus replication in the cell substrate
	Tests for reverse transcriptase or infectivity with retrovirus-sensitive cell line (e.g. *Mus dunni* cells, S^+L^- cells)	Tests for endogenous retrovirus production
Raw material safety assurance (e.g. cell culture components, fetal calf serum)	Virus screen (often by raw material vendor)	Screens out cell culture components with live virus
	Virus inactivation (heat, low pH, γ-irradiation, etc.)	Destroys undetected virus contaminants
Harvest screening	Enumeration of cultivable bioburden for acceptable levels	Screens out harvests with excessive bacteria and fungi
	TEM or Q-PCR	Quantifies endogenous retrovirus load
	In vitro virus screen	Screens out harvests with viruses that grew undetected in the bioreactor
TSE safety	Raw material sourcing from countries not on USDA list	Avoidance of potentially TSE-containing raw materials
	Removal validation	Removal of undetected TSE
Dedicated virus removal steps	Filtration	Sieving of virus from product
	Solvent/detergent	Dissolution of virus envelopes
	Low pH	Denatures virus surface proteins
	Heat	Denatures virus protein
Purification steps that concomitantly remove virus	Protein A chromatography	Virus flows through during loading, prior to product elution
	Anion exchange chromatography	Charge-based separation of viruses from protein products
	Other chromatography procedures	Various

[a] The safety strategy of individual products may vary on a case-by-case basis and do not necessarily possess each element in this table.
[b] HAP, hamster antibody production; MAP, mouse antibody production. TEM, transmission electron microscopy.

spiked solution is subjected to the ensuing manufacturing step or steps under scaled-down laboratory conditions representative of the commercial unit operation. The virus contents of both the input and output material are then measured, most often using infectivity assays, but quantitative polymerase chain reaction (Q-PCR) assays are increasingly being employed. The removal capacity of the studied step(s) is then estimated from the total virus contents of the input and output materials (calculated from the virus titers multiplied by the different volumes of each material) and expressed as a $\log_{10}$ reduction.

In this review, we outline current approaches to assure the viral and TSE safety of plasma-derived products and biotechnology pharmaceuticals (e.g. recombinant DNA products and monoclonal antibodies).

Viral safety of blood and blood-derived products

Blood-derived and plasma-derived products can be divided into two groups: labile blood components and plasma-derived products. Labile blood components (e.g. whole blood, fresh frozen plasma for transfusion, red cell concentrate, platelets, and other cellular elements) are prepared from donations of individuals or small pools of donors, with little if any processing. Viral safely of these products is assured by careful donor selection and monitoring, donation testing and — under some circumstances — recipient monitoring. Nevertheless, some risk of transmission exists during the 'window period' early in infection, when viremia is already present but not detectable by available testing [3]. Currently no viral inactivation and/or removal steps exist for the preparation of labile blood products, although research is under way to develop effective viral clearance methods for platelet concentrates and packed red cells [4]. Unfortunately, however, treatments with most pathogen-inactivating compounds reduce or destroy the normal functions of red cells and platelets. Thus, the development of suitable procedures to clear viruses from labile blood components remains a challenge for transfusion medicine.

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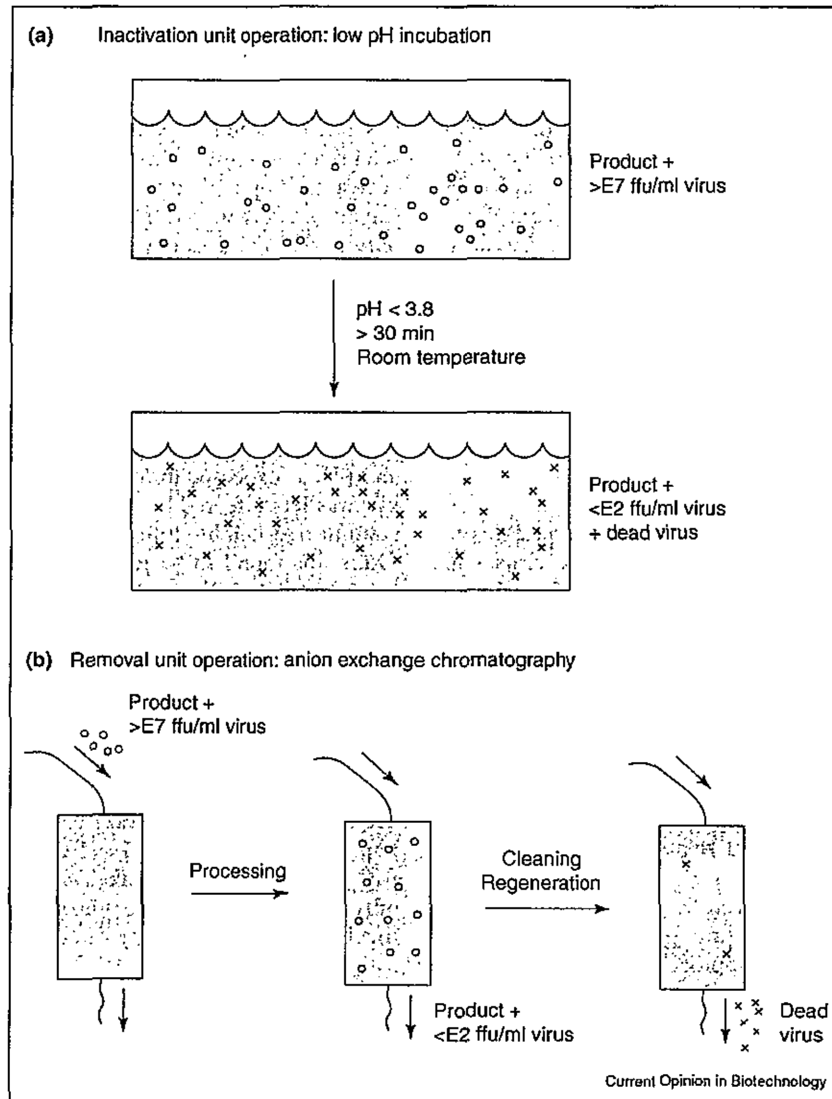
Figure 1



Typical virus clearance validation studies. **(a)** Low pH inactivates some viruses by chemically damaging surface proteins [13]. A typical low pH incubation step for a monoclonal antibody product consists of incubating the product intermediate at pH <3.8 for 30–60 min at room temperature. A typical virus spike would consist of $>10^7$ (>E7 in figure) focus forming units (ffu) per ml, while typically virus would be undetectable after the incubation (assay detection limits are about 10^2 ffu/ml; E2 in figure). The spike recovery would be measured using infectivity assays. Because dead virus particles would still be present, Q-PCR assays would not be suitable for measuring virus clearance. **(b)** Flow through anion exchange chromatography can be used to separate negatively charged viruses such as SV40 or retroviruses from positively charged protein products like monoclonal antibodies [14,19]. A typical monoclonal antibody would flow through an anion exchange column at neutral pH and low conductivity; spike recovery could be measured either by infectivity or using Q-PCR. Virus still bound to the column after antibody flow through would be eluted and destroyed by the high salt and/or alkaline regeneration and cleaning steps.

The second group of blood products includes plasma derivatives such as clotting factors, immunoglobulins, α-1 proteinase inhibitor, and albumin. These are manufactured from large pools of plasma comprising thousands of individual donations. Because of the donor pool size, the risk of viral transmission for these products can be higher than from transfusable units obtained from a single donor or a small pool of donors. Thus, initial screening and testing alone cannot fully assure viral safety of plasma derivatives, which rely heavily on the ability of manufacturing processes to remove and/or inactivate viruses. Currently, plasma derivatives are manufactured by

methods that incorporate specific steps to inactivate viruses (e.g. heat and virus-inactivating chemicals such as organic solvent and detergent) [1]. These methods are highly effective, especially against some clinically relevant viruses such as HBV, HCV and HIV. Separation steps that function primarily to purify products (e.g. Cohn fractionation and similar processes) or are deliberately added to remove pathogens (e.g. viral filters) enhance safety further. Modern plasma derivatives have excellent safety records with regard to enveloped viruses that were major problems in the past (e.g. HBV, HCV and HIV). Available methodologies are less effective in clearing non-enveloped viruses (e.g. human parvovirus B19 and hepatitis A virus) [5]. These viruses are fairly heat stable and not inactivated by currently available chemical methods effective against enveloped viruses. Non-enveloped viruses also tend to be small in size and hence relatively difficult to remove by filtration.

The need for more effective manufacturing strategies to clear non-enveloped viruses and to inactivate viruses in labile blood components has spurred research into developing new and alternative methodologies. Currently, several viral inactivation methods are being investigated using photochemicals (e.g. psoralens, riboflavin, phenothiazines and phthalocyanines) [6], other chemicals (e.g. inactine, β-propiolactone, aldehydes/amines, S-303 and iodine) [7], and UV-C irradiation [8].

Viral safety of recombinant DNA biopharmaceuticals and monoclonal antibodies

Biopharmaceuticals are derived from relatively homogeneous source materials and are purified by multistep schemes. Thus, viral safety approaches are robust, amenable to scientific analysis and have evolved during the past few years (Table 1) [9••]. With the advent of fluorogenic 5′-nuclease-based Q-PCR, a new series of assays became available to measure virus titers in process and validation samples. Q-PCR can be used to quantify surrogate components of the viruses, such as genomic nucleic acids or enzymes like reverse transcriptase [10•], that are more easily measured than infectivity. These assays offer increased precision and sensitivity over standard infectivity assays, and they directly quantify total particle counts rather than complete infectious virions. Q-PCR assays lend themselves to measuring clearance by unit operations that remove viruses (e.g. chromatography and filtration), but not to those that inactivate them (e.g. solvent/detergent or low-pH); infectivity assays can still be used to measure clearance by these unit operations. One particular advantage of Q-PCR assays is that they can easily measure more than one virus in a single preparation; thus, clearance studies can be performed by spiking three or more viruses into feedstock for a single column or filter and the clearance of each virus measured simultaneously in separate assays [11•].

Unit operation robustness is critical for reliable bioprocessing. Certain unit operations were reported anecdotally to be highly robust for virus removal [12] and their mechanism of virus removal or inactivation is known (Figure 1). For some of these operations, robustness has been verified experimentally and described in the scientific literature. Clearance was dependent on a few critical unit operation parameters, but was relatively less sensitive to others so long as these parameters remained within defined acceptable manufacturing ranges. For example, low-pH inactivation of murine retroviruses was, as expected for a chemical reaction, highly dependent on time, temperature and pH, but relatively independent of the type of model protein or salt content in the matrix (Figure 1a) [13•]. Likewise, Simian virus 40 (SV40) capture by flow-through anion exchange (AEX) chromatography was highly dependent on buffer conductivity and pH, as expected for protein binding to an AEX resin, but was less affected by model protein load, flow rate or column bed height (Figure 1b) [14••]. Virus removal by filters was found to be highly dependent on size of the model virus, as expected for size-based sieving, but was less dependent on buffer composition, process time and pressure or on membrane lot and model protein [15]. One filtration parameter with a clear impact on virus removal, at least using one type of virus filter (Viresolve NFP™ membranes), is pressure-adjusted flow rate, a parameter not normally controlled in validation studies. When flow rates declined after extended processing at constant pressure, the $\log_{10}$ reduction values also decreased, perhaps because of changes in effective pore size owing to fouling [16••]. Although this has been demonstrated with only one filter brand, there is no reason to believe that this mechanism may not apply to other filter types.

Regulators are concerned about the robustness of virus removal by chromatography after extensive cleaning and reuse (cycling) of resins [17]. The theoretical concern is that resin degradation or fouling over time might impair viral clearance. Recent studies with protein A chromatography and AEX chromatography, however, found that viral clearance was remarkably stable after even extensive resin cycling. These studies identified useful surrogate performance attributes that changed before, or simultaneously with, reduced viral clearance; for protein A chromatography, decreases in step yield and antibody breakthrough in the flow through appeared to be the most sensitive indicators of degraded column performance [18••]. For AEX columns, increases in band spreading and in back pressure or the appearance of impurities in the process fluid was indicative of the end of their effective functional lifetime [19•] — and loss of their ability to clear viruses.

Overall, rigorous scientific investigations demonstrated that viral clearance achieved by a variety of unit operations was quite robust (e.g. clearance by low-pH inactiva-

tion, membrane filtration, protein A chromatography and AEX chromatography).

Emerging technologies promise to complement the currently available methods for viral clearance. Some emerging technologies are nearing commercialization, whereas others require additional development. Ion exchange membrane adsorbers have ligand–virus-binding properties similar to those of AEX chromatography, but membranes possess certain practical advantages. For example, ligand–target binding to membranes is largely kinetic and not limited by pore diffusion; thus, membranes allow very high flow rates, short processing times and low pressure drops. Membranes are disposable and generally require less floor space and specialized equipment than columns, while their performance validation is simplified because post-use cleaning is not necessary. Ion exchange membranes have already been used successfully to bind and then release virus particles in vaccine production [20•]; removing viruses from process intermediates should be even simpler, as the particles are discarded with the disposable adsorber and not recovered. Thus, it is desirable to develop membranes incorporating ligands and with operating conditions that favor tight interactions between viruses and membranes, because the binding need not be reversible.

Broad-spectrum pulsed light can inactivate a variety of mammalian viruses [21•], but robustness (e.g. interference by high protein concentrations) must be addressed before this technology can be recommended for widespread use in bioprocessing or for the treatment of raw materials. Virus–product partitioning by flocculation followed by microfiltration [22•] and micelle-based extraction [23•] might also become realistic methods at some point, however, significant technical issues, such as maximizing product recovery and improving virus partitioning robustness, must be resolved before these methods become successfully commercialized.

Transmissible spongiform encephalopathies and the safety of blood and plasma products

As noted above, TSE agents have been responsible for hundreds of iatrogenic infections spread via contaminated medical products worldwide, most commonly by hGH and dura mater (i.e. the fibrous membrane around the brain) allografts. Serious concerns have been raised regarding the safety of other medical products, including biopharmaceutical products containing or prepared from human-derived or animal-derived materials, because the TSE agents are relatively resistant to procedures that inactivate conventional microorganisms [24]. Special concern has been expressed about the potential of variant CJD (vCJD), attributed to human infection with the agent of bovine spongiform encephalopathy (BSE). Unlike other forms of CJD, vCJD has clearly been transmitted by transfused blood (non-leukoreduced red cells) from two asymptomatic donors (in the UK); the donations were made 18 months and three years before the donors became ill with clinical vCJD. One recipient developed vCJD six years after transfusion, while a second recipient was neurologically asymptomatic at death, five years after transfusion, but had histological evidence of vCJD infection. Since 1999, US blood donors who traveled or lived in many BSE-endemic areas for substantial periods of time — risk-adjusted for rates of BSE in different countries — have been considered to be at increased risk for vCJD and deferred from donating [25,26,27••]. Products prepared from pools containing plasma from potentially vCJD-infected blood must also be considered to pose a risk, although no transmissions of vCJD have been attributed to plasma derivatives. Leukofiltration only modestly reduced TSE infectivity in the blood of experimentally infected hamsters [28], and more than half of the total infectivity in blood remained in the plasma.

An additional concern is the possibility that TSE agents might contaminate cell substrates used to manufacture biopharmaceuticals, because they have experimentally infected cell cultures of several kinds [29]; the extent to which this risk affects the cell lines used for actual biopharmaceutical production is unclear and currently under study.

Tests for the abnormal prion protein — commonly referred to as PrP^{res} or PrP^{Sc} — may serve as a useful surrogate for infectivity assays in preliminary validation studies, although the nature of the infectious TSE agent itself is still a matter of scientific debate and bioassays remain the current 'gold standard' for detecting residual agent. Several tests of bovine brain tissue have been licensed by the USDA (US Department of Agriculture) for post mortem diagnosis of BSE, although these tests remain negative during the first years of infection. No validated, effective screening assay is yet available to detect TSE agents in living animals or humans [26,27••,30].

Procedures for decontaminating equipment and surfaces potentially exposed to materials containing TSE agents in clinical settings have recently been reviewed [31]; the recommended procedures, although appropriate for healthcare facilities, are generally incompatible with the manufacture of biopharmaceutical products themselves, being so harsh as to destroy the active components. Nevertheless, solutions of sodium hydroxide and some other chemical disinfectants — properly applied — could be satisfactory to inactivate TSE infectivity on stainless steel and some other manufacturing surfaces. Potentially, these disinfectants might even serve to decontaminate those resins that can withstand harsh chemical treatments, although the effects of specific cleaning regimens on contaminated resins have not yet been studied.

Several processes used to manufacture plasma-derived products have been shown to reduce spiked or endogenous TSE agent infectivity [32–35]. In experimental studies, leukoreduction, differential precipitation, membrane filtration, depth filtration, and resin chromatography [28,29,32–36,37••,38,39] removed some PrP^{res} and TSE infectivity from blood and plasma products. These studies, modeled on virus validation studies, typically used plasma or manufacturing intermediates that had been endogenously infected (from blood of experimentally infected animals) or exogenously infected (using some kind of brain-derived material spiked into blood). Spiking studies employed a variety of spike materials (TSE agent strains and tissue preparations), and different detection methods such as western blotting or infectivity bioassays in rodents. 'Spike relevance' is a problem inherent to TSE spiking studies. Because the physical form of the infectious TSE agent in plasma is unknown, it is not certain that brain-derived spiking materials are predictive models; it cannot be confidently concluded from a negative assay for the product of an experimental study that a similar plasma-derived material would necessarily be safe for humans. In addition, amounts of infectivity below the limit of detection in animal studies might still suffice to infect humans. Even a very marked reduction of infectivity in a model TSE validation study does not necessarily prove that a process completely removed all infectivity, although repeated measurements of experimental clearance (i.e. log reduction values computed from multiple tests of animal-adapted TSE infectivity in serially diluted feedstocks and products assayed in rodents) probably provide a reasonable estimate for the effectiveness of a manufacturing process.

There are several practical difficulties in validating the inactivation or removal of TSE agents during manufacturing processes. Progress in this area has been hampered by the lack of rapid, sensitive and accessible tests to detect residual infectivity. The most sensitive detection methods are *in vivo* bioassays, but these are costly and take months or even years to complete. It is also difficult to prove that the removal of spiked infectivity by processes tested in scaled-down pilot models truly represent the effect of the full-scale manufacturing process on endogenous infectivity. Although the methods listed above could prove useful in reducing the levels of TSE infectivity in some biopharmaceutical products, careful source selection remains an important first-line safeguard to minimize the risk of transmission.

Conclusions

The pathogen safety of biopharmaceuticals is ensured by careful screening of the source materials, the inclusion of pathogen clearance steps in the manufacturing process, and end-product testing. In general, plasma-derived products have excellent safety profiles relative to large viruses and an improving safety profile for small viruses. Recombinant DNA and monoclonal antibody pharmaceuticals are prepared with robust manufacturing schemes amenable to engineering principles and continuous improvement. Finally, careful source selection remains the frontline for TSE safety of biopharmaceuticals.

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