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Quality assurance of monoclonal products: virologic and molecular biologic considerations

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SUMMARY

The FDA has set limits concerning the viral and molecular contamination of monoclonal antibody products intended for human use. Industry has an obligation to be as familiar with these limits as it has been with federal requirements pertaining to pyrogens and bacteria. The assessment of risk from polynucleotides, based on molecular biologic and existing technical limitations, is discussed, as is the strategy of validating the purification of monoclonal antibodies of viral contaminants in terms of an indicator organism concept.

INTRODUCTION

Monoclonal antibodies (MA) have a variety of uses in clinical medicine (Table 1). Cooperative efforts between academia and industry as well as independent research efforts have led to the development of potentially therapeutic products. The manufacturing of MA is accomplished by hybridoma technology which was first described in 1975 [8]. Because monoclonal antibodies are considered biologics, their manufacture is regulated by the FDA. The Code of Federal Regulations title 21 (CFR) [4,5] describes requirements and methods for biologics, i.e., mycoplasma, general safety and sterility. Numerous additional proposed standards for safety and efficacy are detailed in FDA 'Points to Consider' documents [6,15] because MA intended for in vivo use are frequently contaminated with adventitious agents that include viruses and bacteria. These organisms are potential vectors of dis-

Table 1

Applications for the in vivo use of MA

(1)	Diagnostic imaging
	(a) normal cells/receptors
	(b) neoplastic cells
(2)	Therapeutics
	(a) neutralization of reptile and insect venoms
	(b) conjugation with bacterial toxins
	(c) tumor specific destruction
	(d) conjugation with drugs with low toxicity thresholds; drug delivery ('magic bullet')

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ease and can be traced to the indigenous population present in hybridoma cell culture, ascites fluid or, less likely, the manufacturing environment. When an infectious agent is known to contaminate the hybridoma stock and that agent is a human pathogen, testing for that agent in crude and final products is a minimal requirement.

In the context of product safety, hybridoma technology is gradually becoming assimilated by industry and regulatory authorities. In our experience, compliance with both Good Manufacturing Practice regulations and FDA 'Points to Consider' documents has been greatest by those pharmaceutical companies with previous experience in good manufacturing practices, good laboratory practices, quality control and quality assurance; university-affiliated research programs, on the other hand, have a poorer record.

However, because of a combination of factors ranging from an urgent need to treat patients to ignorance of regulations, not all manufacturers comply with these requirements or guidelines, and, in some cases, manufacturers perform no testing prior to using MA clinically. When time is of the essence, validation of the manufacturing process, rapid antigen-capture and nucleic acid hybridization techniques are reasonable alternatives to not performing more time-intensive testing of products.

MATERIALS AND METHODS

General safety tests were performed with Swiss Webster albino mice (<22 g) and Hartley strain albino guinea pigs (<400 g). 0.5 ml and 5.0 ml of the MA sample was injected intraperitoneally into mice and guinea pigs, respectively. After injection the animals were observed for 7 days. Guinea pigs were housed individually in cages conforming to USDA requirements. Mice were group-caged. Both species were housed at 20–25°C and 30–70% relative humidity. In addition to dry animal chow, the guinea pig diet was supplemented with either apples, potatoes or cabbage. Guinea pigs unable to properly use the automatic 'lick-it' water system drank from water pans.

MA products were rapidly screened for HIV p-24 core protein antigen by enzyme-linked immunosorbent assay (ELISA) (Abbott HTLV-III EIA Kit, North Chicago, IL). Human immunodeficiency virus (HIV) cultures were made in H-9 cells [12]. Cell supernatants were assayed for p-24 by ELISA 14 days post-infection.

Sindbis virus [20] and infectious bovine rhinotracheitis virus (IBR) (ATCC No. VR188) plaque assays were performed in chick embryo fibroblasts. Viral adsorption was for 1 h at 37 ± 1°C in 5% CO_2 in air. Following adsorption, the inocula were removed and monolayers overlaid with vitamins for Eagle's modified minimum essential medium (MEM), BME amino acids, non-essential amino acids for Eagle's modified MEM, L-glutamine, DEAE-dextran, Earl's salts and glucose and calf serum in a base of Nobel agar. These agar overlay materials were obtained from Flow Laboratories, McLean, VA except for Nobel agar (BBL Microbiology Systems, Cockeysville, MD). Incubation was at 37 ± 1°C in 5% CO_2 in air. Plaques, 3–4 mm in diameter, were visualized by neutral red staining 48 h post-infection.

RESULTS AND DISCUSSION

A review of the specifications describing prohibited viable microorganisms (mycoplasma, aerobic bacteria, yeast and fungi) is shown in Table 2.

Table 2

Safety requirements for biologics[a]

General safety test:	21 CFR 610.11
Sterility test:	21 CFR 610.12 (validation of process is required)
Purity (pyrogens):	21 CFR 610.13
Animal serum additive allergic response:	21 CFR 610.15
Mycoplasma test:	21 CFR 610.30
Hepatitis requirements:	21 CFR 610.40

[a] The requirements listed above for biologics can be found in the Code of Federal Regulations available from the U.S. Printing Office.

Table 3[a]

21 CFR Safety test

Expt. No.	Animal No.	Protein concn. (μg/ml)	Mice (in g)			Guinea pigs (in g)			
			initial wt.	final wt.	change in wt.	initial wt.	final wt.	change in wt.	Overall result
1	1	citrate	15	18.8	3.6	345.1	308.1	−37.0	Fail
	2	buffer	23.8	24.1	0.3	316.5		day 6	
2	1	50	21.8	24.5	2.7	387.4	442.9	35.5	Pass
	2		20.9	25.1	5.2	336.0	380.3	44.3	
3	1	50	18.4	29.8	11.4	290.3	338.5	48.2	Pass
	2		21.7	28.1	6.4	398.6	412.8	14.2	
4	1	50	18.0	16.7	−1.7	323.1	330.1	7.0	Fail
	2		17.1	20.8	3.7	268.7	307.5	38.8	
5	1	50	18.2	28.2	10.0	340.2	398.2	58.0	Fail
	2		16.0	22.9	6.9	358.3		day 1	
							repeat		
						364.2	449.7	85.5	Pass
						367.4	419.8	52.4	
6	1	0.72	21.4	25.8	4.4	324.5	373.8	49.3	Pass
	2		18.7	26.4	7.7	375.7	424.1	48.4	
	control		21.4	28.3	6.9	352.2	355.2	3.0	
7	1	0.54	19.4	28.3	8.9	284.2	300.4	16.2	Fail
	2		19.6	28.2	8.6	283.1		day 3	
	control		18.8	28.3	9.5	294.8	286.2	−8.6	
							repeat		
						354.4	407.4	53.0	Pass
						303.4	371.2	67.8	
						322.9	378.6	55.7	
8	1	0.8	20.3	23.9	3.6	329.9	363.7	33.8	Pass
	2		21.7	25.8	4.1	396.0	427.3	31.3	
	control		20.2	25.3	5.1	364.7	423.9	59.1	
9	1	0.59	18.1	21.1	3.0	395.0	458.5	63.5	Pass
	2		16.0	21.2	5.2	399.5	463.1	63.6	
	control		17.6	22.5	4.9	326.9	399.5	72.6	
10	1	37.9	20.5	31.0	10.5	232.0	281.0	49.0	Pass
	2		21.5	24.2	2.7	345.0	361.0	16.0	
11	1	0.79	18.7	19.0	0.3	333.7	370.0	36.3	Fail
	2		19.0	18.2	−0.8	381.4	413.1	31.7	
	control		18.9	21.2	2.3	385.9	281.9	−104.0	
				repeat					
	1		19.6	24.5	4.9				Pass
	2		19.2	25.2	6.0				
	control		19.4	25.2	5.8				
12 /	1	0.59	19.3	21.8	2.5	271.0	281.5	10.5	Pass
	2		19.2	21.4	2.2	280.0	313.3	33.3	
	control		17.1	20.4	3.3	346.0	404.1	58.1	

Table 3, contd.

Expt. No.	Animal No.	Protein concn. (μg/ml)	Mice (in g)			Guinea pigs (in g)			
			initial wt.	final wt.	change in wt.	initial wt.	final wt.	change in wt.	Overall result
13	1	2.2	20.5		day 4	335.0	365.5	30.5	Fail
	2		20.5		day 7	399.5	444.9	45.4	
14	1	0.68	20.9	24.8	3.9	304.2	317.8	13.6	Pass
	2		20.2	21.9	1.7	286.0	326.9	40.9	
	control		18.0	19.3	1.3	267.9	295.1	27.2	
15	1	0.76	18.4	23.9	5.5	320.1	380.4	60.3	Pass
	2		20.0	23.5	3.5	351.9	376.4	24.5	
	control		20.5	23.8	3.3	324.6	295.1	−29.5	
16	1	0.1	19.5	25.8	6.3	379.3	416.2	36.9	Pass
	2		19.5	23.8	4.3	331.1	372.5	41.4	
	control		20.0	25.8	5.8	367.3	418.2	50.9	
17	1	0.1	21.5	29.2	7.7	355.4	411.9	56.5	Pass
	2		19.9	26.5	6.6	356.5	423.7	67.2	
	control		17.1	22.3	5.2	379.0	421.9	42.9	
18	1	50	21.3	23.7	2.4	354.6	372.0	17.4	Fail
	2		21.7	29.7	7.8	379.8		day 2	
							repeat		
						378.5	425.3	46.8	Pass
						390.1	439.5	49.4	
19	1	50	14.8	19.9	5.1	366.6	343.0	−23	Fail
	2		19.1	22.4	3.3	398.0	419.8	21.8	
							repeat		
						356.0	364.4	8.5	Pass
						377.6	425.2	47.6	
Average weight change			(232.8 ÷ 45) = 5.17 g			(1763.7 ÷ 40) = 44.1 g			

[a] Day indicates when death was observed. The protein concentration is the concentration of the monoclonal antibody in the dose.

These specifications are widely accepted as standard parameters of safety and purity in the United States pharmaceutical and medical device industries. Similarly, the scientific rationale for eliminating pyrogens from drugs and biologics is well known. However, the requirements dealing with non-specific toxins, viruses and polynucleotides are less well known.

21 CFR Safety

The 21 CFR Safety test is designed to detect non-specific toxic factors. In our opinion, it is a test that would benefit by the inclusion of a set of a positive and negative controls. Its criteria for passage include that the animals injected show no signs of toxicity, gain weight, and survive the test period (7 days). We frequently observe (Table 3) cases in which a product that initially fails a CFR Safety test subsequently passes a retest.

In 19 tests of MA products, eight failures were noted (42%). The validity of these data is unknown since all the products passed when retested. In three cases, at the manufacturer's request, a repeat test was not performed. Moreover, we have not ob-

Table 4[a]

Adventitious viral contaminants

Virus	Nucleic acid	Size (nm)
Murine-derived hybridomas [1,2,11,17,18]		
Reo type 3	ds RNA; N	75–80
Hantaan	ss RNA; E	90–100
Polyoma	ds DNA; N	45–55
Pneumonia virus of mice	ss RNA; E	150–300
Mouse adenovirus	ds DNA; N	65–80
Minute virus of mice	ds DNA; N	18–20
Mouse hepatitis	ss RNA; E	75–160
Ectromelia	ds DNA; E	unknown
Sendai	ss RNA; E	150–300
Mouse encephalomyelitis (Theiler's GD VII)	ss RNA; N	22–30
LCM	ss RNA; E	unknown
Mouse salivary gland (murine CMV)	ds DNA; E	100–200
EDIM	unknown	unknown
Thymic	unknown	unknown
LDH	ss RNA; E	150–300
Human-derived hybridomas [1]		
Epstein-Barr virus and EBV DNA	ds DNA; E	100–200
Cytomegalovirus	ds DNA; E	100–200
Retroviruses (HIV)	two RNA strands; E	80–120
Hepatitis B virus surface antigen	ds DNA; E	42

[a] Derived from Ref. 6.

ds = double-stranded; N = naked; ss = single-stranded; E = enveloped.

Table 5

Comparative nucleotide biology of hormones and genes

(A) Hormone gene lengths [9]

	Polypeptide length in amino acid residues	Nominal nucleotide length
Insulin: α chain	30	90
Insulin: β chain	21	63
Corticotropin	39	117
Oxytocin	9	27
Bradykinin	9	27
Thyrotropin-releasing factor	3	9
Enkephalins	5	15

(B) Gene content[a] [9,10]

	Approximate number of base pairs in genome	No. of possible genes
φX174 phage	5.4×10^3	5
T7 phage	4.0×10^4	35
Lambda phage	4.8×10^4	42
T-2, T-4 phage	1.8×10^5	158
Paramyxovirus (measles)	1.8×10^4	16
E. coli genome	4.0×10^6	3500
Homo sapiens	2.0×10^9	200000

[a] Assumes that the average gene in prokaryotes is 1150 nucleotide pairs, and 10000 nucleotide pairs in eukaryotes.

served failures in this test to correlate with positive CFR sterility or positive CFR mycoplasma results. However, in one case, we observed that a product failed the sterility test and passed the safety test.

A more sensitive assessment of non-specific systemic toxicity would include a battery of biochemical and hematologic tests, such as is used in subchronic toxicity tests and in clinical medicine. These tests would detect subtle influences not severe enough to induce weight loss or death. It is unlikely that toxicity severe enough to cause death or weight loss in an animal cannot be detected by examination of blood chemistry. Additional testing of this nature will facilitate interpretation of confusing results.

The murine viruses listed in Table 4 are required to be screened when a MA is of murine derivation. In most cases, a murine antibody production (MAP) test [13] is performed in order to detect whether or not specific pathogen-free mice produce viral antibody after being injected with the MA. The test typically requires 4–6 weeks and is relatively expensive to perform. In vitro direct antigen capture ELISA methods can be coupled to in vitro

Table 6

Screening of monoclonal antibodies for HTLV III (HIV) antigen

The presence or absence of HTLV III (HIV) antigens was determined by relating the absorbance of the specimen to the cut-off value. The cut-off value is the absorbance of the negative control mean plus the factor 0.050. (Specimens with absorbance values less than the cut-off value are considered non-reactive for HTLV III (HIV) antigens by the criteria of the test.) For the run to be valid, the difference between the means of the positive and negative controls should be 0.400 or greater.

Specimen	Absorbance at 492 nm		Comment
1	0.078	0.082	non-reactive
2	0.077	0.080	non-reactive
3	0.140	0.077	non-reactive
4	0.085	0.080	non-reactive
5	0.075	0.065	non-reactive
6	0.096	0.114	non-reactive
7	0.091	0.119	non-reactive
Controls			
negative	(1) 0.095		non-reactive
	(2) 0.089		non-reactive
	(3) 0.144		non-reactive
	mean = 0.109		
positive	(1) 1.387		reactive
	(2) 1.357		reactive
	mean = 1.372		
Cut-off value	0.159		

Difference of positive and negative control means: 1.372 − 0.109 = 1.263

Table 7

Components of process validation: inactivation of viruses[a]

(A) Basic biochemical procedures
1. Salt fractionation of culture or ascites fluids
2. Size exclusion chromatography
3. Affinity chromatography
4. Ion-exchange chromatography
5. Ultracentrifugation
6. Lyophilization
7. Polyacrylamide gel electrophoresis
8. Agarose gel electrophoresis
9. Ultrafiltration

(B) Physical or chemical procedures
1. Elevated temperature
2. pH variation
3. Exposure to:
(a) organic solvents
(b) chelating agents
(c) nucleases

[a] The procedures listed can be used alone or in series to inactivate and/or remove polynucleotides and/or viruses from biological products.

and in vivo enrichment procedures and may offer the prospect of a more rapid and sensitive analysis. ELISA kits for the detection of HIV and hepatitis B surface antigen* are commercially available. The former has proven useful for the rapid screening of therapeutic MA products derived from human hybridomas (Table 6) for HIV p-24. To confirm that the antigen detected is associated with infectious virus the MA is incubated in H-9 cells for 21 days. The supernatant is analyzed for p-24 antigen or for reverse transcriptase enzymatic activity. The relationship between antigen potency and viral infectivity is under investigation.

Validation of inactivation of viruses – indicator concept

Validation of the process used in the manufacture of MA or other biologics must demonstrate the removal or inactivation of adventitious viruses and/or polynucleotides from each bulk lot and/or finished lot. Table 7 lists several routine biochemical and physical purification processes that can be used alone or in series to inactivate or remove polynucleotides and/or viruses.

Validation of the manufacturing process (Quality Assurance) may obviate the need to perform safety and purity tests on the bulk or finished lot (Quality Control). We have used both Sindbis virus (a single-stranded RNA-enveloped virus) and IBR (a double-stranded DNA-enveloped virus) as indicator organisms in 'spiking' experiments (known amounts of virus deliberately inoculated into the biological product) which were designed to dem-

* NML Organon Teknika Corporation, Irving, TX: Qualitative Third Generation Enzyme Linked Immunosorbent Assay for the Detection of Hepatitis B Surface Antigen subtype ad and ay in Human Serum Kit.

Table 8

Spiking experiment

Factor 8 was spiked to 10^9 or 10^8 pfu/ml with Sindbis virus or IBR virus, respectively. The spiked factor 8 preparation was then exposed to the manufacturing process in the sequence: freezing, lyophilization, dry heat. Samples were removed at each of the above process steps and plaque assays were performed with chick embryo fibroblasts. The change in log is calculated from the difference in infectivity relative to the inoculum after freeze-thawing. IBR was completely inactivated by freeze-thawing.

Component of process	Log 10 (pfu/ml)		Log 10 reduction	
	Sindbis	IBR	Sindbis	IBR
Viral inoculum	9.00	8.1	–	–
Inoculum after freeze-thawing	8.20	0	–	⩾8.0
Factor 8 spiked then frozen	7.10	0	0.9	⩾8.0
Lyophilization	6.87	0	1.3	⩾8.0
24 h dry heat (68°C)	3.41	0	4.8	⩾8.0
48 h dry heat (68°C)	2.02	0	6.2	⩾8.0
72 h dry heat (68°C)	<0.04	0	⩾8.2	⩾8.0

onstrate whether or not the manufacturing process [7] either inactivated or removed viruses from a biologic (Factor 8) derived from blood (Table 8).

Sindbis virus (known to be stable at −70°C) was chosen because part of the manufacturing process involved lyophilization. The results indicated that Sindbis virus infectivity was not affected by lyophilization but could be destroyed by dry heat. IBR virus infectivity was, however, rapidly destroyed by freeze-thawing and/or lyophilization. Destruction of IBR, if present in Factor 8, is virtually guaranteed if the manufacturing process has been validated against Sindbis virus. These results (Table 8) suggest that 'spiking' experiments which quantitatively demonstrate the removal or inactivation of more resistant indicator viruses can provide assurance that the final or bulk lot is free of less resistant viruses. Although other applications may require different viruses, experience in the area of disinfection [16] shows that the parvo- and picornaviruses (Table 9) may be useful indicators of inactivation since they are among the most resistant of all viruses to inactivation.

Molecular assessment of risk: polynucleotide mass and chain length

The rationale for requiring that MA contain less than 10 pg of DNA per dose [6] is based on the fact that hybridoma cells are derived, in part, from a malignant cell line (myeloma). Ten picograms is the current limit of detection when nick-translated hybridoma cell DNA is prepared. In our view, emphasis should also be placed on the size and sequence of the contaminating DNA since it is possible that MA products used clinically contain trace amounts

Table 9

Ranking of FDA 'Points to Consider' viruses based on their resistance to disinfection[a]

Agent	Family
Pneumonia virus of mice	Paramyxoviridae
Sendai	Paramyxoviridae
Mouse salivary gland	Herpesviridae
LCM	Arenaviridae
LDH	Togaviridae
Mouse hepatitis	Coronaviridae
Polyoma	Papovaviridae
Ectromelia	Poxviridae
Reo type 3	Reoviridae
Mouse adenovirus	Adenoviridae
Mouse encephalomyelitis (Theiler's GD VII)	Picornaviridae
Minute virus of mice	Parvoviridae

[a] In order of increasing resistance to chemical inactivation. Derived from Prince [16].

of functional DNA. Specifically, genetic control elements and polynucleotides of codegenic length such as transposons should be absent from monoclonal products intended for human use.

In assessing the risk of polynucleotide contamination, it is interesting to survey the size of prokaryotic and eukaryotic genes and proteins (Table 5). Hormones are the smallest biologically active proteins. For example, thyrotropin-releasing factor has a nominal chain length (excluding introns) of only nine nucleotides. However, a typical polypeptide contains 50–2000 or more amino acid residues in a specific sequence. A gene coding for the biosynthesis of a polypeptide chain would therefore have, correspondingly, at least 150–6000 or more nucleotide base pairs. If the average polypeptide chain has approximately 380 residues [9], this would correspond to approximately 1150 nucleotide pairs. Since each nucleotide pair has a molecular weight of about 650, the molecular mass of the above hypothetical gene would be about $650 \times 1150 = 750\,000$ daltons, or 1.2×10^{-6} pg. Thus, the presence of a single typical gene will not be detected until detection systems improve in sensitivity by a factor of about 8 000 000. It may be easier to visualize DNA by microscopic methods. In double-stranded DNA, base pairs are separated from each other by about 0.34 nm. Thus, the length of a gene for an average polypeptide is about 0.4 μm [9].

The genome of mammals and mammalian viruses contain large amounts of DNA for which the function is unknown or uncertain. 'Selfish' DNA, non-sense DNA and introns are thought to be vestiges or consequences of molecular evolution. The theory of the 'selfish gene' [3] states that this type of DNA replicates more than the bulk of the DNA and that, in doing so, is not harmful to its 'host' genome. Thus, from a limits point of view, the presence of 10 pg of 'selfish' or otherwise non-functional DNA in a dosage form is likely to be harmless to the patient.

Movable genetic elements such as those found in retroviruses like the AIDS virus (HIV) behave like transposons. A transposon is a DNA sequence able to replicate itself and insert a copy of itself at a new location in the genome. This event may be mutagenic, preventing or altering normal gene expression. The long terminal repeat postulated to be associated with the mechanism of transposition is composed of three contiguous sequences that are not codegenic for messenger RNA, ribosomal RNA or transfer RNA. The length of the long terminal repeat is approximately 260–1440 base pairs. Transposons in yeast and maize range from 5000 to 6300 base pairs.

Another category of controlling elements are replication origins. DNA replication origins are sequences of DNA, 100–850 base pairs in length, within a chromosome where the initiation of DNA replication begins. If present as a contaminant, they pose a risk of altering gene expression.

In practice, it is possible to separate DNA polynucleotides up to 200 base pairs in length, even if they differ from each other by just one base pair, by polyacrylamide gel electrophoresis. Further, the sequence [11,14,19] of a polynucleotide can be determined by standard radiolabeling, digestion and electrophoretic techniques. Accordingly, a decision can be made on the nature of the risk associated with contaminating polynucleotides. A proposed polynucleotide limit stating that no polynucleotides may be present in a MA in lengths greater than or equal to 100 base pairs may be a proper starting point. Sequence information may also be used to assure that polynucleotides, if present, are not likely to be controlling elements. Thus, the 'Points to Consider' limit of less than 10 pg of DNA per dose is a conservative one if the size, sequence and functioning of the polynucleotides is unknown. However, a MA exceeding the accepted limit may also be safe if its DNA is not codegenic, mutagenic or capable of altering the genetic control of the patient. Notwithstanding the controversial nature of some of the above requirements, manufacturers are obligated from an ethical and legal point of view to manufacture safe products. As such, they should do all that is reasonable to assure that biotechnology products are safe and effective for human use.

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