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Notes to applicants for marketing authorizations on the production and quality control of monoclonal antibodies of murine origin intended for use in man

Committee for Proprietary Medicinal Products: Ad Hoc Working Party on Biotechnology/Pharmacy

1. INTRODUCTION

The fusion of B-lymphocytes from immunized animals with myeloma cells results in the formation of hybrid cells, known as hybridomas, which grow continuously and secrete antibodies. Such cells may be selected, after cloning, for the stable secretion of antibodies of defined specificities. The continuous culture of these cloned cell lines allows the preparation of monoclonal antibodies in large quantities. Monoclonal antibodies produced in this way are available for a variety of therapeutic purposes, such as anti-tumour therapy, immunomodulation and passive immunization, as well as for *in vivo* diagnosis and preparative procedures for biologicals. The requirements set out below are intended to apply to monoclonal antibodies, obtained from hybridomas of murine origin, for therapeutic and *in vivo* diagnostic use in humans. Monoclonal antibodies intended for use in the purification of other products should be shown to be pure and free from adventitious agents by the methods described in this document. Monoclonal antibodies to be used for diagnostic purposes *in vitro* are not the concern of this paper.

Important considerations for the clinical use of monoclonal antibodies of murine origin are the possible unintentional immunological cross-reactivity of the antibody with human tissue antigens other than those desired, and the possible presence of viruses and/or potentially oncogenic macromolecules.

A general problem with the therapeutic use of murine monoclonal antibodies in man may be the induction of antibodies in the recipient against murine immunoglobulin or other proteins present in the product. These may result in adverse reactions and limit

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the duration of effective antibody therapy. It may be prudent to minimize the murine protein load administered to the patient by the use of a parental myeloma cell which does not itself synthesize immunoglobulin chains.

A flexible approach to the control of monoclonal antibodies should be adopted so that requirements can be modified in the light of further experience gained in their production and clinical use. Details of the implementation of these requirements for an individual product will generally reflect the circumstances in which the product will be used, for example, the clinical indication.

2. POINTS TO CONSIDER IN MANUFACTURE

This paper proposes notes for manufacturers of monoclonal antibodies and is intended to help them assemble the information needed to support applications for marketing authorization within the EEC and to establish appropriate control testing.

Several of the requirements relating to establishments in which biological products are manufactured (e.g. revised Requirements for Biological Substances No. 1; WHO, TRS 323: Requirements for Manufacturing Establishments and Control Laboratories) apply to the manufacture of monoclonal antibodies. Attention is drawn to the following points. Production areas should be decontaminated before they are used for the manufacture of monoclonal antibodies. The production of monoclonal antibodies should be performed only by staff whose state of health does not compromise the quality of the product and who are not otherwise involved in work which could result in infectious agents being transmitted to the monoclonal antibody product. Persons not directly concerned with the production processes, other than official representatives of the national control authority, should not be permitted to enter the production area. Particular emphasis should be given to the recommendations contained in Part A, section 1, Revised Requirements for Biological Substances No. 1 (WHO TRS 323, page 13) regarding the training and experience of persons in charge of production and testing and of those assigned to various areas of responsibility in the manufacturing establishment.

Many of the general requirements for the quality control of biological products, such as potency, abnormal toxicity testing, absence of adverse immunological reactions, freedom from inappropriate contaminants, stability and freedom from antibiotics, will apply to monoclonal antibodies. It is undesirable to use in production agents that are known to provoke sensitivity reactions in certain individuals such as, for example, penicillin or other β-lactam antibiotics. In addition, the following points, set out below, should be considered. These apply to monoclonal antibodies obtained from hybridomas of murine–murine origin. Monoclonal antibodies produced by hybridomas of other parentage or by other technologies may require different control measures.

3. SOURCE MATERIAL

Whenever possible, murine tissues and animals used as source materials should be shown to be free of viruses as indicated in Appendix I(a).

3.1. *Myeloma cells*

The source, name and characteristics of the parent myeloma cell line should be given. It should be shown that the cell line is a pure culture of either mouse or rat

myeloma cells and does not contain cells of other origin. Details of the immunoglobulin chains synthesized or secreted by these cells should be included. The myeloma cell line used for fusion should be preferably selected as one that does not synthesize any immunoglobulin chains. Cryopreserved samples of the myeloma cells should be retained in case retrospective investigations become necessary.

3.2. *Immune parental cell*

The source of the immune parental cells, including information on the species, strain and immunization procedure used in the preparation of the donor animal should be provided. The source of immunogen should also be given. If the immunogen is derived from human sources,information as to the health of the donor(s) should be provided. Any relevant clinical data on the donor must be reported.

3.3. *Fusion and cloning*

A complete description of the production of the hybridoma cell line should be provided including details of cell fusion, cloning and recloning procedures. Sufficient data should be given to allow an assessment of the efficiency of the cloning procedure.

If feeder cells are used they should be derived from animals or cell seed stock shown to be free of viruses by testing as described in Appendix I.

3.4. *Identity of hybridoma line*

The characteristics of the hybridoma cell line should be detailed. These should include the specificity, class and, where appropriate, subclass of the immunoglobulin secreted, together with any distinguishing features, such as isoenzyme/immunochemical markers. It should be recorded whether the hybridoma secretes parent myeloma immunoglobulin chains and it should be shown that the cell line is stable in respect of antibody secretion up to and beyond the passage level used for routine production. Appropriate precautions should be taken to avoid cross-contamination with other cells.

3.5. *Monoclonal antibody*

The immunological properties of the antibody should be described in detail, including its antigenic specificity, complement binding and any unintentional reactivity with or cytotoxicity for human tissues distinct from the intended target antigen. Appendix II contains a list of tissues which should be among those tested.

4. CONTROL OF SEED LOT

It is essential that production is based on a clearly delineated seed lot system involving a master cell bank and manufacturer's working cell bank. This should be described in detail and the number of vials of the cell banks available and information on their storage provided. In order to establish the seed lot, cells of the original hybridoma line should be propagated *in vitro* wherever possible and should be confirmed as being either of mouse or of rat origin as appropriate. No other cell lines should be handled simultaneously in the same laboratory or by the same persons during establishment of the seed lots. Information should be provided on the stability of antibody secretion by cells of the cell banks after storage and recovery. Samples of the seed must be retained until at least after the expiry date of the resulting final lot. Evidence that the cells of the seed lot are free from microbial contamination (viral, bacterial, mycotic or mycoplas-

mal) should be provided. Appropriate tests to demonstrate the absence of virus contamination as described under (a), (b) and (c) in Appendix I should be performed. Examination of the cell line by transmission and scanning electron microscopy may provide valuable information on viral contamination. Only in exceptional circumstances should a seed lot containing viruses other than endogenous murine retroviruses be considered for production. Under no circumstances should cell lines contaminated with the viruses listed in Appendix I, Table 1, Group I (which are known to cause disease in man) or polyoma virus, be used for production. The proposed purification procedure used in production of the monoclonal antibody should be shown to be capable of removing and/or inactivating retroviruses and, if present, other viruses.

5. PRODUCTION

It should be clearly stated whether cell culture supernatant or ascitic fluid is being used as the source of antibody. *In vitro* production of monoclonal antibodies at finite passage level offers prospects of a higher degree of control and standardization than *in vivo* production and is the preferred method. Ideally not more than one cell line should be cultivated at the same time in the same production area. However, if other cell lines are cultivated in parallel, records must be kept of the cell lines handled and evidence presented for the absence of cross-contamination between them.

5.1. In vitro *production: finite passage*

A production batch should normally originate from a fresh ampoule of the seed. A description of the cell culture techniques employed in full-scale production cycles should be provided together with the criteria that cell supernatants must meet to be acceptable for further processing (e.g. titre of monoclonal antibody). The maximum permitted number of cell population doublings, starting with a fresh sample of the seed lot (e.g. manufacturer's working cell bank), should be defined and based on information concerning the stability of the hybridoma cell line on prolonged cultivation (e.g. yield of monoclonal antibody; distribution of characteristic cell markers).

Particular attention should be given to the degree and nature of any microbial contamination in culture vessels during culture. If animal serum-derived additives are used, they should be shown to be free from adventitious agents, e.g. calf serum should be free of bovine diarrhoea virus and other potential contaminants. The bulk culture fluid should be shown to be free from mycoplasmal, mycotic and bacterial contamination and should be tested for the presence of viruses using a general test involving inoculation into suitable cell substrates as described in procedures given in Appendix I, test (b). The extent and nature of such testing should be agreed on a case-by-case basis with the National Control Authority.

5.2. In vitro *production: continuous culture*

The control and standardization of continuous culture production pose considerable problems. However, if it can be demonstrated that this method is clearly advantageous and that all aspects are rigorously controlled, it may be considered for production of monoclonal antibodies. In such cases particular attention should be given to stability of the hybridomas during the process and to the yield and integrity of monoclonal antibody obtained. A clear definition of a batch of product should be given and it should be shown that consecutive batches yield a consistent product. Regular testing

for microbial contaminants should be performed and criteria for rejection of harvests and premature termination of the culture should be defined. The period of continuous cultivation should be specified and this should be based on information concerning the stability of the system and consistency of the product up to and beyond this limit. In cases of long-term continuous cultivation, the hybridoma and monoclonal antibody should be completely re-evaluated, as set out in sections 3.4 and 3.5, at intervals based on information concerning the stability of the system and the character of the product.

5.3. In vivo *production*

(a) Each production batch should originate from a fresh ampoule of the seed (e.g. manufacturer's working cell bank). The maximum permissible number of serial passages *in vivo* during normal production should be defined and restricted: justification of this limit should include information concerning the yield of monoclonal antibody and the stability of the hybridoma characteristics on *in vivo* passage up to beyond that used in production. Indefinite passage in animals is not acceptable.

(b) The strain and origin of the animals used for production should be specified, together with their genotype and age. They should be from a closed, specific pathogen-free (SPF) colony which is routinely monitored for those viruses listed in Appendix I, Table 1. The long-term records of the mouse breeding colony in respect of freedom from viral contamination should be considered in relation to the reliability of maintenance of the colony. Evidence should also be presented that animals are maintained under SPF conditions at all times during transfer and use.

(c) The number of animals and the procedure used to prepare the bulk ascitic harvest should be given in detail. Full details should be provided on any substances used to pretreat mice or rats to facilitate growth of hybridomas. Data concerning the titre of the antibody in and storage conditions of the bulk ascitic fluid should be provided (e.g. temperature, duration, details of any proteolytic enzyme inhibitors added). Particular attention should be paid to the degree and nature of any microbial contamination (bacterial, mycotic and mycoplasmal) in the bulk ascitic fluid. Testing procedures capable of detecting all of the murine viruses listed in Appendix I, Table 1 should be performed, as indicated in Appendix I(a) and (b), on at least the first five bulk harvests of the product. However, it may be expected that general testing methods for viruses may be sufficient as experience of production is gained. Consequently, after the first five production runs, general testing for viruses, limited to those described in Appendix I test (b), may be considered adequate. Any infectious agent should be identified and tests for viruses in Appendix II, Group I, Table 1 should be negative. If the source of mice is changed to a different colony or supplier, tests described in Appendix I(a) should be performed on at least the first five bulk harvests to re-establish consistency of freedom from contaminant agents.

6. PURIFICATION OF THE ANTIBODY

6.1. *Methods*

The procedures used to purify the monoclonal antibody from culture supernatant fluid or from ascitic fluid should be described in detail. It is important to ensure that

purification procedures do not impair relevant immunobiological features of the immunoglobulin. Consideration should be given to incorporating procedures which inactivate potential viral contaminants where such methods will not compromise the biological activity of the product.

6.2. *Validation of the purification process*

The capacity of the purification procedure to remove contaminants, including unwanted immunoglobulins, DNA, viruses and irritants (pristane) used in the generation of ascites should be demonstrated as should the reproducibility of the purification process in successive production runs. Pilot scale studies using a representative collection of viruses possessing a range of biophysical and structural features (including retroviruses) or radioactively labelled compounds (e.g. DNA) with which the crude preparation is 'spiked' should be performed. A reduction factor for such contaminants at each stage of the purification process, and overall, should be defined and reported by using, if necessary, concentrations of DNA and viruses in excess of that expected during normal production. Any inactivation process used should be shown to be effective and not compromise the biological activity of the product.

7. THE BULK FINAL PROCESSED PRODUCT

7.1. *Criteria of purity*

Precise criteria for purity of the monoclonal antibody product, especially with respect to protein and DNA components, should be provided.

Analytical tests should include, for example, column chromatography (including HPLC), isoelectric focusing, SDS PAGE under reducing and non-reducing conditions, and the DNA assay method.

Specific limits should be set for each contaminant, including DNA and proteins that may be derived from the parental cell, host animal, tissue culture medium or manufacturing procedures. The DNA content, particularly for DNA of hybridoma origin, should be assayed by a sensitive and reliable assay method, the characteristics of which should be described in detail. Currently, the DNA hybridization technique is the most sensitive method.

Specifications, with limits, should be given for the specific activity, i.e. antibody activity per unit weight protein. To establish the standardization of the appropriate assay, a reference batch of the monoclonal antibody preferably produced *in vitro* and purified as thoroughly as possible, should be prepared.

7.2. *Freedom from adventitious agents*

The final bulk product should be shown to be free from bacterial, fungal and mycoplasmal contamination and should be tested for viruses according to Appendix I, Table 2. Evidence should be presented to show that any viral contaminant known to be present in the bulk harvest has been eliminated or inactivated.

7.3. *Reference preparation*

Material from an early batch which has been or will be clinically evaluated should be retained as a working reference preparation for purity and potency for comparing future batches.

In addition a highly purified monoclonal antibody preparation made by an *in vitro* culture method should be established (see 7.1) to permit the determination of specific activity.

8. TESTS ON FINAL DOSAGE FORM

The final containers should be shown to comply with the existing general EEC requirements, for example for sterility, pyrogenicity and abnormal toxicity, etc.

Particular attention should be given to assessment of the degree of aggregation or molecular fragmentation of the immunoglobulin. All possible steps should be taken to prevent aggregation. At least 95% of the immunoglobulin present should be in the form of molecular monomers or dimers. Any departure from this limit should be justified.

9. CONSISTENCY

Evidence should be provided on the consistency of production, for example, on at least five consecutive full-scale production batches. This should include information on the bulk harvest and final dosage form as well as on in-process control tests.

10. MODIFIED MONOCLONAL ANTIBODIES

For some purposes it may be desirable to modify monoclonal antibodies (e.g. by conjugation with a toxin, radiolabelling or attachment to specific drugs for 'targeting'). For such products additional specific control procedures will be required but these are dealt with best on a case-by-case basis. In addition to a detailed description of their preparation and purification, general requirements for such products might include information concerning the half-life of the monoclonal antibody, of the drug or toxin, and also of the conjugate after injection into a recipient. Information about the cross-reactivity of the conjugate, as well as of the antibody itself, would be required, as would data concerning the toxicity and stability of the conjugate. Modification of the antibody could potentially change its antigenic specificity and this should be examined in detail with particular reference to the presence of any unintentional reactivity of the modified antibody with human tissues (Appendix II).

For some applications sub-fragments of antibody, e.g. Fab or $F(ab')_2$, may have advantages. Where such fragments are preferred for clinical use, their molecular and antigenic properties should be defined. If $F(ab')_2$ or Fab is used, specified limits for impurities, for example fragments other than those desired or intact immunoglobulin, should be defined and should comply with definitions of existing immunoglobulin preparations. The information described under section 7.1 should be provided. Upper limits should be set for residual levels in the product of enzymes used in such processes (e.g. pepsin or papain).

11. PRE-CLINICAL SAFETY TESTING

In general, classical toxicological studies in animals may be of only limited relevance to studies of monoclonal antibodies. However, limited safety testing will usually be required for most products.

It is important that pre-clinical safety testing should include adequate studies for the presence of unintentional reactions of the antibody with human tissues (see Appendix II).

Reference should be made to separate guidelines on pre-clinical safety testing of products derived from the new biotechnologies.

APPENDIX I

Table 1 lists viruses which should be considered as potential contaminants in the manufacture of monoclonal antibodies. Viruses for which evidence exists of a capacity to infect man or primates are to be found in Group I. Those viruses for which there is no evidence of infection in man but which could nevertheless pose a potential danger, for example in immunocompromised individuals, are listed in Group II. Testing for virusus should be performed in laboratories with experience in routine virus testing and should be performed in accordance with good laboratory practice. Table 2 lists the tests for viruses to be performed at the different stages of production.

Testing for viral contamination

(a) Tests for detection of specific viruses listed in Table 1, for example Mouse Antibody Production (MAP) or Rat Antibody Production (RAP) tests or other tests of at least equivalent sensitivity and reliability. Additional specific tests may need to be carried out for lymphocytic choriomeningitis

TABLE 1. Potential contaminants in the manufacture of monoclonal antibodies

Group	Virus	Species affected
I	Hantavirus (Haemorrhagic fever with renal syndrome)*	M, R
	Lymphocytic choriomeningitis virus (LCMV)*	M
	Rat rotavirus*	R
	Reovirus type 3 (reo 3)*	M, R
	Sendai virus*	M, R
II	Ectromelia virus*	M
	K virus (K)	M
	Kilham rat virus (KRV)*	R
	Lactic dehydrogenase virus (LDH)	M
	Minute virus of mice (MVM)	M, R
	Mouse adenovirus (MAV)*	M
	Mouse cytomegalovirus (MCMV)	M
	Mouse encephalomyelitis virus (MEV, Theiler's or GDVII)	M
	Mouse hepatitis virus (MHV)	M
	Mouse rotavirus (EDIM)	M
	Pneumonia virus of mice (PVM)*	M, R
	Polyoma virus	M
	Rat coronavirus (RCV)	R
	Retroviruses*	M, R
	Sialodacryoadenitis virus (SDA)	R
	Thymic virus	M
	Toolan virus (HI)*	R

M: mouse; R: rat.

* Known to be capable of replicating *in vitro* in cells of human and monkey origin.

TABLE 2. Testing scheme for viral contaminants

	Appendix I sections which are applicable		
Hybridoma (seed lot)	(a)	(b)	(c)
Mouse breeding colony	(a)		
Ascitic fluid harvest	(a)*	(b)	
In vitro bulk harvest		(b)	
Bulk final processed product	Specified tests of (b) if virus contamination was detected in the bulk harvest		

* It is proposed that these tests should be carried out on at least the first few production runs, e.g. five.

virus (LCMV), mouse cytomegalo virus, mouse rotavirus (EDIM), thymic virus and lactic dehydrogenase virus. Tests capable of detecting murine retroviruses should be included, for example the XC plaque assay or the S^+L^- focus assay for the detection of ecotropic or xenotropic retroviruses, respectively.

(b) Inoculation of cell cultures capable of detecting a wide range of murine, human and bovine viruses. Examples of useful cell types (substrates) are: murine fibroblast culture, e.g. mouse embryo cultures; human fibroblast cultures, e.g. human diploid cells such as MRC5; continuous cell lines of human, murine and bovine origin. Tests for retroviruses, as under (a), should be included.

(c) Tests in animals for adventitious agents should include the inoculation by the intramuscular route of each of the following groups of animals with the test material or with disrupted cells from the seed lot propagated beyond the maximum level (or population doubling, as appropriate) used for production:

two litters of suckling mice, comprising at least 10 animals less than 24 h old
10 adult mice
five guinea-pigs.

Test material should also be injected intracerebrally into each of 10 adult mice.

The animals should be observed for at least four weeks. Any animals that are sick or show any abnormality should be investigated to establish the cause of illness. Test material can be considered to be suitable for production if at least 80% of the animals inoculated remain healthy and survive the observation period and none of the animals shows evidence of the presence in the tested material of any adventitious agent.

Fertilized eggs may also act as useful substrates. Test material should be injected into eggs by appropriate routes, the chorioallantoic membrane, amniotic cavity and yolk sack of each of 10 embryonated chicken eggs, 9–11 days old. The embryonated eggs should be examined after not less than five days incubation. The allantoic fluids should be tested with guinea-pig and chick or other avian red cells for the presence of haemaglutinins.

APPENDIX II

Suggested list of human tissues to be used for immunohistochemical or cytochemical investigations of cross-reactivity of monoclonal antibodies. This list should reflect the specificity of the antibody and its particular use.

(1) Tonsil, thymus, lymph node.
(2) Bone marrow, blood cells.
(3) Lung, liver, kidney, bladder, spleen, stomach, intestine.
(4) Pancreas, parotid, thyroid, para-thyroid, adrenal, pituitary.
(5) Brain, peripheral nerve.

(6) Heart, striated muscle.
(7) Ovary, testis.
(8) Skin.
(9) Eye.

APPENDIX III: GLOSSARY

1. Murine

'Murine' means derived from an animal belonging to the Muridae family which includes mice and rats.

2. Seed lot

Master cell bank. This is the original cell line on which production is based and may not necessarily have been produced by the manufacturer.

Manufacturer's working cell bank. A homogeneous suspension of seed cells derived from the master cell bank by a finite passage level, aliquoted into individual containers for storage (e.g. in a liquid nitrogen refrigerator). All containers are treated identically during storage and once removed from storage, the containers are not returned to the seed stock.

3. Production method

Production at finite passage. This cultivation method is defined by a limited number of passages or population doublings that must not be exceeded during production.

Continuous culture production. During production, the number of passages or population doublings are not restricted from the beginning. Criteria for the termination has to be defined by the manufacturer.

4. Bulk harvest (intermediate batch)

This is a homogeneous pool of individual harvests (e.g. of all supernatant or ascitic fluids) which is processed together in a single manufacturing run.

5. Bulk final processed product (final batch)

Thus is the finished product, after completion of the manufacturing process, obtained from a bulk harvest of the supernatant or ascitic fluid. It is maintained in a single container and used in the preparation of the final dosage form.

The generation of this final batch has to be clearly defined and unambiguously recorded by the manufacturer.

6. Final dosage form

The finished product is formulated and put into sealed containers which hold the liquid or freeze-dried product in its final dosage form. The containers of a filling lot are processed together and are uniform in their contents and biological potency.