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NOTE FOR GUIDANCE ON THE DEVELOPMENT OF VACCINIA VIRUS BASED VACCINES AGAINST SMALLPOX

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This Note for Guidance will be updated as further experience is gained in the development and manufacture of second generation smallpox vaccines.

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This Note for Guidance will be updated as further experience is gained in the development and manufacture of second generation smallpox vaccines.

1. Introduction

In 1980 the World Health Organisation (WHO) declared the eradication of smallpox. This had been achieved by the use of a vaccine containing vaccinia virus, a strain of poxvirus distinct from smallpox (variola) but which provides cross-protection against it. Vaccine usage and production had declined significantly before 1980 however and many of the currently existing stocks are over twenty five years old. The production methods in use at the time are less acceptable for quality reasons than when smallpox was a major disease. Furthermore, the vaccine was associated with a significant level of adverse reactions of varying severity, including deaths, and was never subjected to a controlled clinical trial to determine its field efficacy accurately. Effectiveness was proven by the fact that the disease was eradicated. With the perception of possible bioterrorist threats there is now renewed interest in smallpox vaccines for emergency use.

During the eradication programme, most vaccine was manufactured on the skin of live animals although there was some experience in the use of embryonated eggs and primary tissue culture derived from chick embryos, rabbit kidneys and bovine kidneys. Animals used in production included calves, sheep, buffaloes and rabbits. Rabbits in particular were used for the production of seed materials and the preferred animals in Europe or the USA for vaccine production were calves or sheep. Renewed production on the skin of live animals has the advantages that it is proven to give rise to effective vaccines and could be initiated quickly as the systems are documented. However, the current acceptability of the process and quality issues of the product with respect to contamination with microbiological agents pose a significant problem. Consequently, there is considerable interest in the production of a 'second generation' of smallpox vaccines using tissue culture systems or embryonated hens' eggs.

Vaccinia vaccines prepared in embryonated hens' eggs were used in large scale during the eradication in South America and are the standard commercial vaccine in Israel. Experience of vaccine produced in cell culture is limited. Vaccine was prepared on chick embryo fibroblasts (CEFs) in Japan before the eradication of the disease. The vaccine had a good safety profile, but its effectiveness is less well documented. As vaccinia has been used as a vector for the expression of antigens, including many HIV trials, there is considerable experience at producing small lots suitable for clinical use by modern methods, notably on CEFs, on human diploid MRC-5 cells and on the continuous African green monkey kidney cell line, Vero. While the immune responses to the vectored genes have been intensively monitored, the response to vaccinia itself is less well documented and there is little or no experience of the effectiveness of cell culture grown vaccines against smallpox itself.

The attainable quality in terms of purity and freedom from unwanted agents is far higher for a cell culture grown vaccine than for vaccine produced on animal skin. However, the safety and efficacy of the vaccine will depend on the quality of the virus seed itself as well as on any contaminants present. Despite a higher microbiological quality, it is noteworthy that a tissue culture grown vaccine may be associated with similar serious adverse events as the animal derived vaccines used most widely in the eradication programme. Indeed, production on tissue culture supposes adaptation of the virus strain to that cell substrate and the effect of

such an adaptation on the immunogenicity and safety of the vaccine should be studied and the properties of the product in animal models established.

2. Scope

This Note for Guidance (NfG) is intended to provide advice regarding the manufacture of, and preclinical and clinical development programmes for, smallpox vaccines that are produced by means of growing vaccinia virus in tissue culture or in embryonated hens' eggs. The tissue culture system may comprise primary cultures, diploid cells or continuous cell lines.

The NfG is also intended to form the basis of assessments by regulatory authorities of the quality, safety and immunogenicity of smallpox vaccines that have been prepared by such methods. Thus, the NfG describes the data that would be required in order to support an application for a Marketing Authorisation in the EU. Guidance regarding the quality and the pre-clinical and clinical evaluation of smallpox vaccines is covered in separate sections of this NfG, although there may be overlaps between the sections.

For the purpose of this document, smallpox vaccines, manufactured by growing vaccinia virus by one of the above methods, have been denoted as *second generation smallpox vaccines*. They will contain replicative virus and are administered by scarification.

This NfG does not apply to smallpox vaccines that are prepared by growing vaccinia virus in live animals. For requirements governing first generation smallpox vaccines, manufacturers are referred to the WHO Requirements for Smallpox Vaccines[1]. Future generations of smallpox vaccines are likely to include viruses produced by genetic engineering and/or gene transfer technologies. These also are excluded from this NfG although many of the points raised here may be applicable.

A number of general guidelines and requirements are relevant to production, quality control and clinical testing of second generation smallpox vaccines and reference should be made to them. These include:

- ICH, WHO and Ph. Eur. guidance and requirements on the use of cell substrates[2-4]
- Ph. Eur. requirements and veterinary guidance on extraneous agents[5-10]
- Ph. Eur. requirements for the use of chicken flocks in vaccine production[11]
- Draft CPMP note for guidance on the use of bovine serum[12]
- CPMP/CVMP TSE note for guidance[13]
- CPMP note for guidance on preclinical pharmacological and toxicological testing of vaccines[14]
- CPMP note for guidance on clinical evaluation of new vaccines[15]

Where applicable, such guidance and requirements must be adhered to.

3. Vaccine strain selection

3.1. History of strains

Many vaccinia strains were used in the eradication programme and their relationship to each other is not clear.

- Lister strain: this strain was developed at the Lister Institute. It is sometimes referred to as the Elstree strain (or Lister/Elstree) since the institute was located at Elstree in the UK. A master seed stock was established by The National Institute of Public Health (RIVM) in The Netherlands in collaboration with WHO and is still held by RIVM. It was prepared by two passages on calf lymph from the original stock established in 1961 on sheep lymph. The master seed has been sent to centres in Paris, Tokyo, Atlanta (CDC) and Moscow. Material from this stock was also provided to manufacturers worldwide although many of them prepared independent

seeds from their own production lots. It was reported to the WHO that 23 out of 59 vaccine producers used this strain in the eradication era.

- New York City Board of Health (NYCBOH) strain: this strain was used in the Americas and in West Africa. Seven vaccine producers reported to WHO that they had used this strain. The EM-63 strain used in Russia is a derivative of the NYCBH strain and was widely used in the eradication of smallpox in India.
- Other vaccinia strains:
  - The Paris strain was reported to have been used by seven vaccine producers in four continents. Other strains were very widely used, including the Copenhagen strain, Bern strain, and the Temple of Heaven strain (possibly more immunogenic) in China.
  - Non replicative strains:
    Note that non-replicative vaccines are not within the scope of this guideline.
    - Modified Virus Ankara (MVA) strain: this strain was an experimental strain created by high level passage on chick embryo fibroblasts (CEFs) and resulted in a highly attenuated (limited replication in human cell culture) strain. It was used in Germany in several hundred thousand recipients as a priming vaccination to alleviate adverse events. The effectiveness of this strain under epidemic conditions is not known; however, attenuated strains warrant further research and development for possible use in individuals with impaired immunity.
    - Other strains, such as NYVAC.

3.2. Availability of strains

Manufacturers should investigate the availability of strains.

- Lister strain: a master seed stock is held at the RIVM; requests for this should be made in the first instance to the WHO.
- Requests for the NYCBOH strain should be made to the Center of Disease Control (CDC) in the USA.
- Information on the access to and the availability of other strains could not be obtained whilst preparing this Note for Guidance.

3.3. Factors affecting choice of strains

The safety profiles of the different strains are reported to vary and comprehensive comparative data are very limited. More documented information on vaccine related adverse reactions is available for the Lister and the NYCBOH strains than for other strains used in the eradication programme.

Since cell culture production and clonal selection might lead to altered characteristics of the virus, it is difficult to provide clear guidance on strain selection. However, the following factors should be considered:

- The safety profile and biological characteristics of the parental strain,
- The presumed effectiveness (based on eradication success) of a vaccine made using the parental strain, assessed by take-rate and field usage,
- The history of the parental strain,
- Growth characteristics of the chosen strain in the cell substrate,
- Whether a manufacturer had prior experience of the strain in the production of a first generation smallpox vaccine.

3.4. Standard reference materials

An International Standard (produced on the flanks of sheep) derived from the Lister strain of

virus is available from the National Institute for Biological Standards and Control (NIBSC), UK. This material is only available in small amounts and is intended for the establishment of in-house potency reference materials. Likewise, there are small amounts of material equivalent to the International Standard for anti-smallpox serum available from NIBSC, which again is available for the establishment of in-house standards.
It is also possible that other National Control Laboratories can provide suitable National Standards, calibrated against the International Standard, for the calibration of in-house reference materials. Given the renewed interest in smallpox vaccines and the noted decline in reference materials, it is highly likely that standards will be developed to replace those currently available as stocks of the latter are further reduced. Potential producers should enquire about the availability of such new reference materials.

4. Quality

4.1. Vaccine Seed Lots

4.1.1. Preparation of vaccine seed lots

A vaccine seed lot system involving a master seed lot and a working seed lot should be employed. Their establishment, storage conditions and life expectancy should be fully described. It should be prepared by passage of the selected vaccine strain in the cell substrate to be used in vaccine production, or *in ovo* if *in ovo* production is planned. It is well recognised that prolonged repeated passage of a virus strain in tissue culture or *in ovo* may reduce its immunogenic property and so the number of passages from the parental virus strain to the master and working seed lot should be limited and justified. In the production of the final lot of vaccine, the number of passages of the vaccine virus from the working seed lot shall not exceed that used for production of the vaccine shown in clinical studies to be satisfactory, unless otherwise justified and authorised. Vaccine should be produced from the working seed lot with a minimum number of intervening passages.
The preparation of the master seed lot from the parental virus strain may include plaque purification or some other form of clonal selection. Plaque purification may help to remove potential adventious viruses present in a parental vaccine strain and render the population more homogeneous. However, it may also select an atypical sub-population.
Characterisation of the master seed lot in a variety of systems, including *in vivo*, will be necessary.

4.1.2. Characterisation of seed lot material

The master seed lot should be characterised as fully as possible. This should include as complete a documented history of the parental strain as possible. Full characterisation against the parental virus strain is preferred, although it is recognised that only a limited amount of the parental strain may be available for this purpose. The characterisation should include the following, unless otherwise justified:

- antigenic analyses using specific antisera and/or monoclonal antibodies,
- biological studies such as infectivity titre, pock formation on CAMs, *in vitro* yield and *in vivo* growth characteristics in a suitable animal model,
- genetic analyses such as restriction mapping/southern blotting, PCR analyses and limited sequencing studies,
- phenotypic and genotypic stability upon passage in the substrate to be used for manufacture,
- neurovirulence testing (see section 5),
- immunogenicity studies (see section 5).

The extent of testing will be dependent on the background history of the originator strain, the history of the cells used for its preparation, the application of clonal selection and the nature of any reagents used in the preparation of the seed lot.

The virus titre of the master and working seed lots should be determined and the virus titre of the working seed lot should be monitored during storage to ensure consistency of vaccine production.

4.1.3. Testing for adventitious agents

The passage history of the parental virus strain and of the cell substrates used to generate the seed lots should be taken into consideration in performing the tests for adventitious agents. Historically, sheep, goats, calves, water buffalos, rabbits, and humans in the 19th century, have been used to propagate the virus. However, the complete passage history of a virus strain is unlikely to be known and any strain may have been passaged through more than one species. For example, the WHO seed stock of the Lister strain held at RIVM was derived on calf lymph from a stock originally derived on sheep lymph.

The general method for testing a live viral vaccine strain for the presence of contaminating viruses is to neutralise the vaccine virus and test for adventitious viruses both *in vitro* and *in vivo*. *In vitro* tests should be performed on at least three cell types, which include the cells to be used for vaccine production, a human cell line and a simian cell line broadly sensitive to virus infection. It is recognised that vaccinia virus is very difficult to neutralise to the extent required for such studies. Furthermore, virus stocks may contain phenotypically distinct intracellular and extracellular forms, the former being more readily neutralised than the latter. If the vaccine virus to be tested is diluted to a lower titre prior to neutralisation, in order to achieve full neutralisation, it should be noted that this may compromise the sensitivity of detection of adventitious viruses.

General testing should be performed according to the Ph. Eur. requirements[5-9]. Additional testing will be required, which may include nucleic acid amplification techniques for specified viruses and reverse transcriptase assays for retroviruses. In the event that complete neutralisation of the vaccine virus cannot be achieved, more emphasis will be placed on alternative methods of detecting specific adventitious contaminants. Testing for specific viruses should also take into account the susceptibility of the production cell substrate (see 4.2, 4.3 and 4.4).

Where materials of ruminant origin are used in preparation of the master and working seeds, compliance with the CPMP/CVMP TSE note for guidance[13] is required.

Sterility, mycobacteria and mycoplasma testing should be in compliance with the Ph. Eur.[16-18]

In the preparation of the master seed, consideration should be given to procedures which would help remove extraneous agents. Since removal or inactivation of such agents is unlikely to be possible at any level of the production process of a live smallpox vaccine, the presence of extraneous agents in the seed lots is not acceptable.

4.2. Cell banks

A cell bank system should consist of a master cell bank and a working cell bank. The cell banks should be established and characterised according to current CPMP/ICH[2] guidelines and WHO[3] and Ph. Eur.[4] requirements.

The potential of the cell substrate to infection by pathogens potentially present in the virus seed should be investigated. This latter point would be highly dependent on the history of the parental vaccine strain and reference should be made to 4.1 above.

For the preparation of live vaccines, the cell line must not be tumorigenic at any population doubling level used for vaccine production.

4.3. Primary cell cultures

Primary cell cultures are not bankable and so the controls and characterisation tests performed on diploid and continuous cell lines are generally not applicable to primary cultures.

Although the main emphasis of the guidelines and requirements referenced in section 4.2 is related to bankable cell lines, guidance on the use of primary cell cultures is provided as an appendix in the CPMP/ICH Note for Guidance on Cell Substrates[2] and there should be adherence to the guidance provided.
Of paramount importance in the use of primary cell cultures is the avoidance of adventitious agent contamination. Thus, the source tissues used in the preparation of the primary cultures must be obtained from closed, specified pathogen free (SPF) healthy flocks or colonies kept solely for the purpose of preparing primary cell cultures. Such flocks or colonies must be stringently controlled for the presence and maintenance of the SPF status at regular intervals. Flocks of chickens used as a source of primary cultures must comply with Ph. Eur. requirements.[11]
The potential of the primary cell cultures to infection by pathogens potentially present in the vaccine virus should be investigated. This latter point would be highly dependent on the history of the parental vaccine strain and reference should be made to 4.1 above.
There is considerable experience in the manufacture and use of measles and mumps live attenuated vaccines produced on chick embryo fibroblast primary cultures and, for additional guidance, manufacturers making use of CEF cultures are referred to the WHO Requirements for measles, mumps and rubella vaccines[19], especially section A.4.1.3 on the derivation of avian-embryo cell cultures.

4.4. Embryonated hens' eggs

An important feature in the use of embryonated hens' eggs is the avoidance of adventitious agent contamination. Thus, the eggs used must be obtained from closed, specified pathogen free (SPF) healthy flocks kept solely for the purpose of seed preparation or vaccine production. Such flocks must be stringently controlled for the presence and maintenance of the SPF status at regular intervals in accordance with Ph. Eur. requirements[11]. Only controlled eggs from such flocks should be used for production of live attenuated smallpox vaccine.
The potential of embryonated hens' eggs to infection by pathogens potentially present in the vaccine virus should be investigated. This latter point would be highly dependent on the history of the parental vaccine strain and reference should be made to 4.1 above.

4.5. Vaccine production

In general, the production of second generation smallpox vaccines is likely to follow that of other live viral vaccines involving minimal downstream processing and the basic requirements for manufacture and control of these smallpox vaccines will be essentially the same as for other live vaccines.
The production of the vaccine should be described in full. Cells from cell banks, primary cells from mammalian or avian origin and/or embryonated eggs should comply with appropriate guidelines and requirements[2-4, 11] as mentioned above. All materials used during production should be described and be of suitable quality. Approved animal serum may be used provided that residual levels have been reduced to acceptable limits. Draft guidance on the use of bovine serum should be consulted[12]. Material of animal origin should comply with the CPMP/CVMP TSE guideline[13]. Penicillin, all other β-lactam antibiotics and streptomycin should neither be used during production nor added to the final product.

4.5.1. Vaccine Virus Growth

On the day of inoculation of a production cell culture with the vaccine virus, the control cells should be examined for cytopathic effects (CPE). Where possible, the production cells should similarly be examined. If any CPE is observed, the batch of cell cultures should not be used

for vaccine production.

4.5.2. Single Virus Harvests

The method of harvesting the vaccine should be described. Vaccinia virus is present in both intracellular and extracellular forms, and the protocol for harvesting should take into consideration that the intracellular form of the virus will need to be released from the production cell culture or the embryonated egg. Typically, single virus harvests will be pooled and from these virus pools, the final bulk will be prepared. However, depending on the unit size of production, one or more of these stages may not be necessary.

An identity test should be performed on the harvested virus. The virus titre should be determined after any filtration or clarification step using either the chorio-allantoic membrane assay (CAM assay) expressed in pock forming units/ml or by a validated cell titration method on cell culture and results expressed in $CCID_{50}$ (cell culture infectious dose) or plaque forming units. A reference preparation should be included to validate the titration assay. Minimum acceptable titres should be established for use of a single virus harvest in the preparation of a virus pool or final bulk.

Tests for extraneous agents should be performed on each single harvest according to the Ph. Eur[5-10] and should be designed to take into account the fact that vaccinia virus is difficult to neutralise. Wherever possible, test systems should be chosen that are refractory to vaccinia virus but capable of detecting potential extraneous agents. Other techniques that are capable of specifically removing/inactivating vaccinia virus to allow extraneous agents to be preferentially detected can also be used. Sensitive molecular biology methods could be used as alternatives to test for the presence of specific viral genomes. The nature of any specified viruses being sought should take into account the method of production, viz. bankable cell culture, primary cells or embryonated eggs. All test systems will need to be properly validated and detection limits for potential pathogens reported and justified.

Controls cells should be assessed for adventitious agent contamination by microscopic examination for CPE and by other assays as specified by the Ph. Eur[5,6,8]. For production based on embryonated eggs, control eggs from each batch used for production should be assayed for the presence of haemagglutinating agents and avian leucosis viruses[7].

Single virus harvests should also be subject to sterility tests and tests for mycoplasma according to the Ph. Eur[16,18].

4.5.3. Virus Pools

The strategy for pooling of single virus harvests into virus pools should be described. Only single virus harvests that comply with the tests outlined in Section 4.5.2 should be used. A virus pool should be subject to a clarification step and may be concentrated to obtain the required virus titre. All processing of the virus pool should be described in detail.

Virus pools should be subject to sterility tests according to the Ph. Eur[16].

4.5.4. Final Bulk

The final bulk can be prepared from one or several virus pools or it may be derived from a single virus harvest. Only single virus harvests and virus pools which comply with the tests outlined in Sections 4.5.2 and 4.5.3 should be used.

4.5.5. Formulation

The following points should be taken into consideration in formulating the final bulk. Substances such as diluents or stabilisers or any other excipient added to the product during preparation of the final bulk should have been shown not to impair the efficacy and safety of the vaccine in the concentrations employed. Freeze-drying for long-term storage is recommended. For reconstitution of freeze dried vaccine, glycerol in the reconstitution fluid

serves to stabilise the vaccine and promotes the adherence of the vaccine to a bifurcated needle or vaccinostyle, a bifurcated needle being the preferred route of administration. The addition of a colorant in the reconstitution medium could be considered, provided it does not impair the safety, the potency and in-use stability of the reconstituted vaccine.
Addition of antibiotics as antimicrobial preservatives is not normally acceptable. For multidose preparations, the need for effective antimicrobial preservation should be evaluated taking into account possible contamination during use and the maximum recommended period of use after opening the container or reconstitution of the vaccine. If an antimicrobial preservative is used, it should not impair the safety or efficacy of the vaccine.

4.5.6. Testing of Final Bulk

The titre of the final bulk should allow for titre loss during filling, freeze drying and shelf life.
Qualitative and quantitative analyses on final bulk vaccine should include tests for total protein content, tests for added substances, a test for residual animal serum proteins (e.g. BSA) and values should fall within acceptable specifications.
Depending on the type of substrate used (i.e. continuous cell lines), there may be a need to develop appropriate and adequately validated tests for residual host cell protein and DNA.
Each final bulk should be tested for sterility according to the Ph. Eur[16].
The final virus bulk should be tested for neurovirulence by an appropriate animal model, such as the intracerebral inoculation of mice, to demonstrate product consistency with respect to its biological phenotype. Neurovirulence testing can also be performed at the virus pool stage. A reference preparation, which may be a previous final bulk or virus pool, may be included. The need for the test will be reviewed in the light of accumulating experience.

Until filling into final containers, the final bulk should be stored under conditions that have been shown to retain the activity of the virus.

4.5.7. Filling and Containers

The filling and labelling of containers should comply with the principals developed for all biological products as provided in the EU Good Manufacturing Practices for Biological Products[20].

4.6. Control Tests on Final Vaccine Product

After reconstitution, samples of containers from each batch of final vaccine should be subjected to tests for sterility, identity and potency. For vaccines prepared on embryonated hens' eggs, endotoxin levels should fall within limits based on the results of production batch analyses.

4.6.1 Potency assays

The WHO established a titre for the first generation vaccine of not less than $1x10^8$ pock forming units per millilitre. This should serve as the basis for the titre of second generation vaccines. Final titre to be set up as release specification should be justified by the preclinical and/or clinical data obtained for the developed vaccine.
For determination of potency, the chorio-allantoic membrane assay expressed in pock forming units/ml or a validated cell titration method on cell culture with results expressed in $CCID_{50}$ (cell culture infectious dose) or plaque forming units should be used. A reference preparation should be included to validate the assay.

4.6.2 Abnormal Toxicity

A general safety test should be in place to demonstrate that there is no abnormal toxicity associated with the final product. In line with current Ph. Eur. practices, this test need only be

performed during the process validation period.

4.6.3 Stability

An end of shelf life specification should be defined and adequately justified. Maintenance of potency throughout the period of validity should be demonstrated. Any loss of potency during storage should be assessed and excessive loss even within the limits of acceptable potency may indicate that the vaccine is unstable. For a freeze dried vaccines, appropriate thermal stability testing criteria (e.g. 4 weeks at 37°C) should also be developed.

4.6.4 Consistency of Production

Data from successive production lots should demonstrate consistency of production and be used to set limits on relevant parameters.

5. Preclinical

5.1. General principles

As a general principle, the pharmacological and toxicological characteristics of a candidate vaccine should be investigated with a comparator which should be the original vaccine strain from which it was developed. The latter should have been manufactured in compliance with the WHO Requirements of 1965.

Smallpox (variola) cannot be easily tested in animal models. In contrast, vaccinia is able to protect against smallpox and other orthopox viruses pathogenic in mammals. Regarding selection of relevant species for animal testing, vaccinia is known to cause relevant immunogenicity in several species (mouse, rabbit, monkey).

Toxicity testing will be required for the master seed lot, the working seed lots and the final product. Pharmacodynamic studies will be required on the final product.

In the present document animal testing is recommended in models that have been only recently described in the literature. This is a growing field of research and newer, more appropriate models may become apparent. The use of all animal models should be fully described and justified to take account of such developments in the area. Applicants are recommended to use these models in close collaboration with an institution that has shown its competence in this field, in order to avoid time-consuming set-up of complex models to demonstrate their proficiency.

5.2. Pharmacodynamics

Assessment of the protective effect of smallpox vaccines cannot be evaluated in man either in formal efficacy trials or in challenge testing in vaccinated individuals with pox viruses. Therefore the assessment of likely protective effect must depend to a certain extent on appropriate studies in animals. These tests are however a prerequisite before envisaging any clinical trials.

5.2.1 Primary pharmacodynamics

The primary endpoint of the animal studies should be the protection by the candidate vaccine in comparison with an original vaccine against the challenge with a relevant pathogenic orthopox virus.
Preclinical testing of second generation smallpox vaccines, even in relevant animal models, can only partly replace clinical studies in man. Any animal model to be used should be as close as possible to the human setting. Cross-protection should be demonstrated against two different pathogenic orthopox viruses in two different mammalian species. A stepwise approach is recommended using a non-primate model during early preclinical, pharmaceutical development. Protective activity in the mouse is a prerequisite for the final product before envisaging clinical trials. The final confirmation of protection of the final product should be investigated in monkeys and may be obtained in parallel with the first clinical studies in humans.

- The BALB/c mouse can be used as a non-primate model and is well described in the scientific literature. The primary endpoint is protection against a lethal respiratory infectious dose of challenge with orthopox virus e.g. cowpox virus. Symptoms induced by the challenge virus such as effect on body weight can be used as secondary endpoints. The results should be at least equivalent to the results observed with the comparator. Additional data on protective activity can be derived from dosing studies and challenge with viruses with different virulence. The intradermal route, preferably

by scarification, is recommended as the vaccination route.
- One monkey model is well described in the scientific literature, i.e. the Cynomolgus model challenged by a monkeypox virus aerosol. The primary endpoint is protection against a lethal dose. This requires a biosafety level 3 laboratory, BSL3.

Additional data that could be collected from the BALB/c mouse and cynomolgus models:
- induction of pustule and scar ('vaccine take')
- induction of antibody response and cell-mediated immune response:
  Analysis of antibody responses could be determined by measuring neutralising antibody titres. Analysis of cell-mediated response could be determined by measuring specific CD4 and CD8 subset activities (e.g. by IFN-gamma ELISPOT assay).
- Assessment of viral load:
  Viral load can be assessed by cell titration or genomic quantification

5.2.2 Secondary pharmacodynamics/Safety pharmacology

The effects on respiratory and cardiovascular systems can be investigated in monkeys, preferably in the animals used for primary pharmacodynamics. Effects on central nervous system will be covered by studies on neurovirulence as discussed below.

5.3. Pharmacokinetics

Pharmacokinetic studies are not applicable for this viral vaccine

5.4. Toxicity studies

General toxicity

Virulence

The virulence is thought be dependent on the local replication at the site of entry and diffusion to the blood. The animal model used to assess virulence should be well described with respect to pathogenesis and the outcome measures to be qpplied. Local replication may be investigated in the mouse model based on intradermal injection of mouse ear pinnae and by assessing dose-dependent survival after aerosol application.

Neurovirulence

There is no established model to evaluate the neurovirulence of smallpox vaccines <hich is directly applicable to human post-vaccinal encephalitis.
Neuropathogenicity is determined by the ability to cross the blood-brain barrier (neuroinvasiveness) and by local replication in the brain; these two phenomena have to be tested independently in different models.

The potential to cross the blood-brain barrier could be tested by using the intranasal administration route in the mouse model in order to produce viraemia. Presence of the virus in the brain must be associated with encephalitis markers, such as local increase in TNF- and IL1-mRNA. Direct entrance into the brain via the ethmoid route should be considered especially in case of early encephalitis.
The potential of the vaccine virus to replicate in the brain should be tested by direct intracerebral administration e.g. in young mice.
For both models it is recommended to consider histological damage, replication of virus in the brain tissue and immunogenicity in the same animals.
To evaluate and validate the outcome of the neurovirulence testing it is recommended to use a positive control material, such as an orthopox strain with a high reported neurovirulence, e.g.

Western Reserve strain of vaccinia virus.

Reproductive function
From historical data it is known that vaccination during the first trimester of pregnancy in humans might induce miscarriage and malformations, whereas later during pregnancy the risk of damage appears not to be higher than in untreated women. Reproduction toxicity may be caused by the effects of the immune response to the vaccine or by replication and entrance of the virus to the foetus. Care will be taken not to administer smallpox vaccines to women who might be pregnant.
For emergency situations, as there might be a need to treat women in their early phase of pregnancy while the risks of vaccination during pregnancy are not fully clear, it is recommended to do specific studies with the aim to identifying a possible “high-risk window” during the early phase of pregnancy, which would help in making strong contra-indication of the vaccination at a given period. The reproductive toxicity might be investigated in animals, e.g. mice or rabbits following intradermal application of candidate vaccine or comparator test vaccine. An appropriate study design should consider administration of a single dose of the vaccines to distinct animal groups a few days before or a few days after mating. Addition of groups with single dose administration at later time points during pregnancy should be considered.

Mutagenicity and carcinogenicity
Tests to investigate mutagenicity and carcinogenicity are not necessary.

Local tolerance
Local tolerance should be evaluated with the final product, e.g. in rabbits. Local toxicity will be observed before pock formation. In some cases the potential local effects can be evaluated in single or repeated dose toxicity studies thus obviating the need for separate local tolerance studies.

6. Clinical

6.1. General considerations for the clinical development programme

Under normal circumstances, the clinical assessment of a novel vaccine should include:

- Assessment of immune responses to the major antigen(s)
- Trials to evaluate protective efficacy
- Documentation of the safety profile of the vaccine, including local reactogenicity and early and delayed systemic adverse effects

Since smallpox does not currently exist in the population, trials of protective efficacy are not possible. Therefore, the likely protective efficacy of a new smallpox vaccine must be inferred from other parameters.

Before the global eradication of smallpox, the formation of an appropriately sized pock with subsequent crusting and scarring at the site of primary inoculation correlated with protection against infection. In particular, the surface area of the scar, as well as the number of scars from previous immunisations, showed an inverse relationship with the case-fatality rate. After successful vaccination, the duration of protection was thought to be at least three years, with at least some degree of protection likely persisting for 10 years or more.

Following primary vaccination, a small central lesion (pock or ulcer) of as little as 1-8 mm diameter was reported to be associated with a maximal level of neutralising antibody, although details of study methodologies are not always available. The correlation between lesion size and antibody levels determined by haemagglutination inhibition (HAI) tests appeared to be much weaker. These findings applied to both the NYCBOH (grown in calf lymph or in eggs) and to the Lister-Elstree vaccine strains.

Based on these observations, it is considered that the likely protective efficacy of a novel smallpox vaccine could be inferred from the proportion of vaccinees in whom appropriately sized pocks are achieved at the site of inoculation. Although the predictive value of laboratory assessments of serological and cell-mediated immune responses is unproven in man, past observations and data from studies in animals indicate that such tests should be performed. The correlation between the results of such tests and pock formation should be explored.

The safety profile of those smallpox vaccines that were used up to the time of cessation of routine vaccination following the global eradication of the disease was well described. Serious and life-threatening adverse reactions appeared to occur rarely or very rarely. Nevertheless, the current absence of circulating variola virus carries implications for the risk - benefit relationship of vaccination. Indeed, at present vaccination is usually confined to those working in research facilities where poxviruses are handled.

Thus, the following sections are based on the consideration that the number of subjects to be exposed to a novel smallpox vaccine in clinical trials should be kept to the minimum necessary to provide adequate assurance regarding its likely protective efficacy and safety. Guidance is provided regarding the possible design of clinical trials, the immunological studies that should be performed, and the assessment of safety.

6.2. Assessment of immune responses

6.2.1. Endpoints

6.2.1.1 Presence and dimensions of the skin reaction (pock)

Extrapolating from past experience with vaccines that were used in the global eradication programme, it would be expected that a novel smallpox vaccine would induce a distinct pock in at least 95% of healthy recipients after primary immunisation. The definition of a pock "take" should be based on the appearance of an erythematous papule or pustule at the site of inoculation within one week of vaccination.

It is essential that all lesions should be fully characterised according to their appearance, dimensions (in comparison with a graduated scale) and time of first appearance. The times to crusting (which provides an estimate of the total duration of virus shedding) and to crust fall should be reported. Although patients may be able to record much of this information on pre-formed cards, there should be sufficient visits incorporated into the trial schedule for visual assessments by investigators. Routine photographic recording of lesions should be performed.

6.2.1.2 Immunological responses

Both the humoral and cell-mediated immune responses should be characterised and the correlation between the results of such tests and pock formation should be investigated. An attempt should be made to validate the relationship between immunological parameters and pock formation.

Assessment of the humoral immune response should include the detection and titration of neutralising antibodies using the intracellular mature virion (IMV) against an appropriate reference material calibrated against a suitable standard. However, it is recognised that there is currently a need for the development of appropriate international standards. If more recent technologies (including ELISA tests) are used, they should be validated against the results of neutralisation tests and should differentiate IgG and IgM responses.

Assessments of the cell-mediated component of the immune response should include the evaluation of CD8 T-cell activity using sensitive methods, such as cell activation by live virus and interferon (IFNgamma) production (i.e. by ELISPOT and flow-cytometry)

6.2.2. Design of clinical trials

6.2.2.1 Pharmacological trials

Uncontrolled trials in small groups of healthy adult volunteers should suffice to characterise preliminarily the safety and immunogenicity of the new vaccine. However, depending on the pre-clinical findings, it may be appropriate to perform comparisons between vaccines that contain different plaque forming units per ml (PFU/ml) of vaccinia virus at this stage.

Eligible subjects should have no history of smallpox vaccination. In cases where there may be doubt about previous exposure to vaccinia or other orthopoxviruses, pre-inoculation immunological tests that have a high sensitivity to detect CD4 memory cells (e.g. lymphoproliferation tests) should confirm their naivety. Every effort should be made to exclude subjects with risk factors for the development of an adverse reaction to a live attenuated vaccinia virus vaccine. Special care should be taken to exclude those with any history of atopy (not just eczema) and/or a current active skin disease.

These early trials should be of a sufficient size so as to give an indication of the percentage of vaccinees likely to develop pocks. Lesions should be fully characterised. Data should also be obtained regarding the immunological responses, with sampling at approximately 4 to 6

weeks after inoculation. The numbers to be exposed in these preliminary trials and the timing of the immunological assessments should be justified.

6.2.2.2 Confirmatory immunogenicity trials

These trials should evaluate the new vaccine in larger numbers of subjects. However, subject selection criteria should be similar to those employed in earlier trials. That is, it is not expected that children or elderly subjects would be eligible, and any person with an identifiable risk of an adverse reaction to a live attenuated vaccinia virus should be excluded.

If the results of preliminary trials in man indicate that different PFU/ml vaccines should be compared in larger numbers of subjects, these comparisons should be randomised according to the dose of PFU administered and should be double blind.

Ideally, a randomised and double blind trial that aims to demonstrate non-inferiority between the novel and a licensed vaccine should be carried out. The choice of δ should be justified. In order to maximise the safety database for the novel product, it may be appropriate to employ unbalanced randomisation such that the majority of subjects exposed receive the unauthorised vaccine. If more than one suitable vaccine were to be available, a comparison with one of these would likely be sufficient, provided that the choice of comparator was justified (taking into account factors such as the viral strain and the dose). In all the types of trials described, laboratory studies of immune responses (as detailed above) should be assessed in at least a subset of subjects, depending on the total number to be exposed.

- In the absence of a vaccine that meets current production standards, whether or not licensed in the EU

There are concerns regarding the use of vaccines that do not meet current production standards, except in emergency situations. Therefore, in the absence of an acceptable and/or licensed vaccine in the EU, and based on considerations of the risk-benefit relationship in the absence of circulating variola virus, a comparative trial against an unlicensed vaccine would not be mandatory.

As stated in 6.2.1.1, past experience strongly suggests that the vaccine should elicit a distinct pock in at least 95% of vaccinees. In an uncontrolled trial, the precision of the estimated percentage of recipients who develop a pock should be calculated. The proposed number of subjects to be enrolled should take into consideration the pre-clinical findings and the expected percentage of recipients who will develop pocks based on results of the previous exploratory trials.

- Comparison with a vaccine that meets current production standards, whether or not licensed in the EU

If such a smallpox vaccine were to be available and /or were to be authorised in the EU before commencement, or in the early stages of, the clinical development program of a new vaccine, it would be preferable that some comparative data should be provided. The choice of an appropriate comparator for such a trial should be discussed with the regulatory authorities.

6.2.3. Duration of follow-up of immunity

Currently, the need for, and optimal timing of, sequential doses of vaccine is not clearly established. In addition, past recommendations for previous vaccines may not be applicable to novel products.

An initial application for a marketing authorisation may well occur when less than one year

has elapsed since the majority of subjects studied were exposed to the new vaccine. It is expected that protocols for confirmatory trials of immunogenicity should plan to repeat laboratory tests of immune responses over a much longer period in at least a cohort of subjects. However, due to the lack of information at the current time, it would not be expected that protocols should plan for the administration of sequential doses.

Detailed plans for these assessments should be made available at the time of initial authorisation. It should be expected that adherence to these plans would be among the post-marketing commitments.

6.3. Assessment of safety

The safety profile of several of the previous smallpox vaccines was well known. Several risk factors for various types of adverse reactions have been well described in the literature. Depending on the total number of subjects enrolled in the immunogenicity studies, it may be necessary to expose larger numbers of subjects to the vaccine for the evaluation of safety. In determining the numbers that should be exposed, companies should consult historical data on the reported frequencies of adverse reactions.

It is recognised that pre-authorisation clinical trials would not include a sufficient number of subjects to be able to detect rare or very rare adverse reactions, such as encephalitis. However, the size of the safety database should be sufficient at least to estimate the frequency of uncommon reactions. The opportunity should not be missed of evaluating the characteristics of pock formation in these additional vaccinees, but studies of immunogenicity would not be required. The duration of follow-up for assessment of safety should be at least 3 months for all subjects exposed at the time of an initial application for marketing authorisation, so as to capture the late development of neurotoxicity and any cases of progressive vaccinia.

Unfortunately, the recognised complications of smallpox vaccination, whether or not they are directly attributable to replication of the attenuated virus, cannot be wholly prevented by taking careful note of the medical histories of recipients. There is currently a lack of validated treatments for vaccinia-related complications, which is a major reason for recommending that the total number of subjects studied in trials should be kept to the minimum necessary. Nevertheless, protocols should include information concerning any possible rescue treatments for such complications, based on current information and the availability of potentially beneficial medicinal products. In all cases in which specific treatment of complications is attempted, the therapeutic measures should be carefully recorded and the outcomes monitored and documented.

In addition, in cases of prolonged or severe fever after vaccination, which may represent the occurrence of a viraemia, attempts should be made to document the existence of a viraemia using established and/or experimental virological methods. These patients should be carefully followed.

6.4. Post-authorisation studies

Because of the circumstances surrounding the development of these vaccines, it is likely that initial marketing authorisations would be granted under exceptional circumstances, pending the satisfactory completion of any ongoing studies and/or contingent on the applicant making several post-marketing commitments.

Although vaccine-related adverse events with an onset more than three months after vaccination would not be expected, there should be pro-active monitoring of recipients over a longer time that should be justified. In addition, as already mentioned, it would be expected that protocols should plan for long-term follow-up of immune responses in at least a cohort of recipients.

Ultimately, and depending on the findings in cohorts of subjects who undergo long-term follow-up of immunological responses, it may be appropriate to compare pock formation rates and immunological responses in these subjects with a cohort that is naïve to vaccinia virus.

If not done previously, and depending on the results from trials in healthy adults, it may be justifiable to perform studies of safety and immunogenicity in healthy children and healthy elderly subjects at this stage.

In an emergency situation, subjects who would not normally be given live vaccinia virus (such as pregnant women, the immune compromised and atopic subjects) may need to be vaccinated. Protocols should be in place so that in this specific situation important data regarding pock formation, immunogenicity and safety are captured in these subjects. Given the circumstances that would surround such a situation, applicants would likely draw up such protocols in conjunction with health authorities.

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