Biosafety Advisory Council

15-07-2005

Secretariat

O./ref.: WIV-ISP/BAC/2005_SC_263
Email: bac@sbb.ihe.be

Title: Advice of the Belgian Biosafety Council on the notification B/BE/04/BV1 of the company Pfizer-Animal Health for deliberate release in the environment of genetically modified organisms other than higher plants for research and development

Context

The notification B/BE/04/BV1 was submitted by Pfizer to the Belgian Competent Authorities in May 2004 for a request of deliberate release in the environment of genetically modified organisms other than higher plants for research and development according to Part B of Directive 2001/18/EC and the Royal Decision of 18 December 1998.
The dossier has been officially acknowledged on September 8, 2004. A group of scientific experts were mandated by the Belgian Biosafety Council to evaluate it and their work led to ask complementary information to the notifier. The answers of the notifier to the questions of the experts were received on 9th and 30th of June 2005 and were reviewed by the coordinators of the group.

The planned activity is a clinical trial in cats with two genetically modified herpesviruses designed to vaccinate cats against the infection with the feline immunodeficiency virus (FIV).
The title of the study is: **"Evaluation of the safety of Feline Herpes Virus, bivalent deleted live vaccine, administered as intranasal vaccine to cats".**
The vaccine is a mix of 2 live recombinant attenuated feline herpes viruses (FeHV-1) expressing two genes of the FIV.
The cats will be treated in a veterinary clinic in Mechelen and in 2 breeding catteries located in Sint Katelijne-Waver and Schaerbeek.

Scientific evaluation

The group of experts of the Biosafety Council answered a list of questions which were mainly based on Annex 2 D1 of the European Directive 2001/18/EC and its guidance notes

(2002/623/EC) relative to the risk assessment of genetically modified organisms. The evaluation of the experts is summarised below.

1. The non-modified parental organism (feline herpes virus 1 or FeHV-1 from the group of alphaherpesviruses) occurs worldwide. It is widespread in cat population and causes severe upper respiratory disease. The virus is restricted to the Felidae family and is transmitted by contact with oro-nasal-conjunctival secretions. It is considered as non-dangerous for other animal species and man. It is poorly resistant to external conditions and survives only a few hours in the environment. It has been classified as biological risk of level 2 for animals[1]..

2. The vaccine involved in the trial is bivalent. It includes two FeHV-1 modified viruses, each bearing a different insert from the FIV genome. Both recombinant viruses were obtained by deleting the thymidine kinase (TK) gene from the FeHV-1 vector and replacing it with either the *env* or the *gag* gene of FIV. The deletion of the TK gene is known to induce a drastic attenuation of the herpes virus, which becomes no more able to cause the rhinotracheitis. As expected, no clinical signs suggestive of rhinotracheitis were observed in cats vaccinated with the attenuated herpes virus containing one or the other FIV. All the cats tolerated the vaccine, remained active, continued to eat and gain weight and were presented as healthy animals after vaccination.

3. The genetic modification has been well characterised for the purpose of evaluating the risks (direct, indirect, delayed, immediate, cumulated) to the target organism, the human health and the environment. The genetic stability of the recombinant viruses was satisfactorily addressed through successive backpassages in susceptible cats. However, a comparative sequences analysis of the TK and foreign genes before and after sequential passages in cats would reinforce the safety evaluation of the vector. The notifier has provided the nucleotide sequences of both regions overlapping either *gag* or *env* genes and their flanking regions, including the insertion site in the vector. Comparative analysis of the TK and foreign genes sequences before and after backpassages in cats is also available, showing the perfect match of TK and foreign genes sequences before and after backpassages in cats. Moreover, the stability of the recombinant viruses has been adequately documented. On the other hand, it is assumed (and supported by literature) that the TK deletion is the actual cause of the attenuation. Complementation studies and revertant virus analyses would definitively exclude the hypothesis that other genetic modifications could account for the attenuation character. Requiring such complementary studies is likely too demanding for a clinical trial but the point could be addressed in a possible future marketing authorisation.

4. Direct attempts to detect the presence of the FIV gene product in the FeHV recombinant vector have not been made. However the data to date do not support the presence of a

[1] Animal pathogens of Class of Risk 2 are defined as micro-organisms that can cause disease in animals and present, at different levels, one or other of the following characteristics: limited geographical importance, no or weak interspecific transmission, no vectors or carriers. The economic and or veterinary significance is limited. There is usually effective prophylaxis or treatment available.

functionally active protein in the recombinant viruses. The strongest evidence for the lack of a functional FIV envelope protein within the recombinant herpesvirus is the inability of the FeHV-*env* to infect primate cells in vitro, whereas wildtype FIV is known to do so.

5. Alphaherpesviruses are not particularly suited to long-term survival outside of the host. Since the genetically modified (GM) virus has no advantage over the wild type or parental virus to survive in the environment, this limits its dispersion by vaccine spill or by shedding from oro-nasal-conjunctival secretions during the days following the vaccination.

6. Alphaherpesviruses, including some TK deleted strains, can establish a latent infection and possibly further be reactivated. To address this point, the notifier performed a study showing that latent TK negative FeHV-1 (the GMO) could not be reactivated nor re-excreted after glucocorticoid treatment. One expert considers that this study suggests that the vaccine virus is not present under latent form. Other experts consider that this possibility should be further investigated, as well as the possibility of finding the vector in the blood lymphocytes of vaccinated cats. However, none of the experts consider that these investigations should be necessarily conducted prior to the trial. Instead, they recommend paying a special attention to the putative reactivation of the viruses during the course of the proposed trial, through regular swabs (see below).

7. The disinfection of the surroundings places where the cats will be vaccinated, in order to avoid and/or minimise the spread of the GMvirus beyond the site of release/treated animal, is not unanimously considered as sufficient by the experts. However, no spreading to humans is expected and the probability of spreading to the environment is very low. The possibility of spreading from the vaccinated cat to other cats is not excluded by the experts but, if it occurs, this event would be transient and/or not very efficient. For this reason, keeping the animals isolated during two weeks after vaccination is considered as a satisfactory precautionary measure[2]. This is in contrast with the initial notifier's intention since it clearly states that in order to mimic what would be the vaccinal scheme under field conditions there is no intention to isolate household cats after vaccination (see the recommendation, under conclusion).

8. The risk of the GMvirus to reconvert to his wild type form is not totally excluded since the vaccine is administered intra-nasally, i.e. the natural site of wild type FeHV-1. The recombination could putatively take place in vaccinated animals during either primary infection or reactivation. However, an infection with a wild type virus is not expected to occur in the vaccinated population, reducing the probability of finding the vaccinal viruses together with wild type viruses. Alternatively, the recombination could putatively take place in surrounding animals. The risk that this could lead to virulent recombinants has been evaluated as very low. Keeping the animals isolated during two weeks after vaccination is considered as a satisfactory precautionary measure.

[2] FeHV-1 is excreted by the nasal and ocular secretions over about two weeks after a primary infection.

9. The waste treatment is considered adequate for vials and applicators. Since the shedding of viruses by the vaccinated cats is known to be located in the oropharyngeal sphere and there is no evidence of excreta from vaccinated cats being positive, the waste in cats' litter does not need to be treated.

10. The Council requested a monitoring plan to the notifier, taking into account the potential shedding, recombination and latency of recombinant viruses. The plan proposed by the notifier foresees:

- *Monitoring of shedding – Spread of the recombinant virus:*

Intra nasal swabs from cats in catteries will be collected every 3 days, from 6 days to 27 days after vaccination for the first 10 cats enrolled in the study. The collected swab will be analysed for virus recovery.

The experts ask for a rational justification of the testing period, which could need to be extended (see below, under Recommendations). The position of the company, underlying the difficulty of applying the monitoring to owners' cats, is an additional reason for restricting the clinical trial to cats kept in a controlled environment (catteries, veterinary clinics or practices), as recommended in the conclusion.

- *Monitoring of recombination:*

The notifier believes that the monitoring of a potential recombination is not necessary under field conditions.

According to the experts, this opinion has to be qualified. Indeed, it has been demonstrated that herpesviruses are recombigenic both in vitro and in vivo. Since the vaccine is administered intranasally, the probability of coinfection is not negligible in a same individual already infected with FeHV-1 because the anterior respiratory tract is the natural site of this virus. Therefore, a monitoring of a potential recombination under field conditions makes sense (see the recommendation, under conclusion).

- *Monitoring of latency:*

The notifier believes that a monitoring of latency is not necessary under field conditions.

This opinion is not totally shared by the experts. They highlight that although some TK-alphaherpesviruses cannot be reactivated from a neuronal latency site, it was shown that at least the bovine herpesvirus 1 (BoHV-1) deleted in the TK gene, was still able to be reactivated by induction with dexamethasone. Spontaneous reactivation has not been shown. It must be emphasized that BoHV-1 and FeHV-1 share many pathogenic properties in their respective host species. The limited experiment carried out by the applicant suggests that the putative TK- FeHV-1 reactivation and re-excretion does not occur or at a low rate. However, it would be advisable to monitor the possibility of reactivation and re-excretion under field conditions over a longer period (see below, under Recommendations).

Conclusion

Based on the scientific assessment, the Biosafety Advisory Council concludes that the risk of using this GMvirus in this clinical trial is low and restricted to cats but could still be reduced if some extra precautions are taken. A particular measure recommended by the experts is to keep all the vaccinated animals inside during minimum 2 weeks following vaccination. The Council considers however that implementation of this measure cannot be guaranteed for individual owners. Therefore, the dossier receives a positive advice under the following conditions:

1. The treatment is only administered to cats kept in a controlled environment (catteries, veterinary clinics or practices) guaranteeing that i) the treated cats will be kept contained and ii) will not be in contact with other felidae. The cats that have been administered the GMOs must be maintained in this controlled environment for a minimum of 2 weeks following vaccination.

2. The monitoring as it is proposed by the notifier, i.e. intra nasal swabs collected every 3 days, from 6 days to 27 days after vaccination for the first 10 cats enrolled in the study should be justified on a rational basis. Moreover, it should address the putative recombination and latency issues. Therefore, the notifier must commit himself to collect additional swabs over a determined period in order to monitor the putative re-excretion of vaccine virus. Swabs positive for the presence of a Herpesvirus should be analysed in order to discriminate putative recombined rFeVH/wildtype isolates from the vaccinal and/or the wildtype parent strains. Since most nasal swabs collected soon after vaccination are expected to be positive for rFeVH*env*/*gag*, not all early swabs must necessarily be tested. The Notifier is thus asked to propose a timetable covering an adequately justified period post vaccination, which includes a molecular testing of given positive samples selected on a rational basis. The number of cats to be tested, the number and schedule of swabs per animal and the extent of the testing period should be based on the expected frequency of wildtype FeHV reactivation, in order to have a reasonable probability of isolating any putative re-excreted Herpesvirus . Moreover, any cat presenting unexpected clinical signs that could be related to a FeHV infection must be swabbed. The resulting sample must be carefully analysed for the presence of vaccinal/wildtype/recombined FeHV strains. Should a recombined isolate be detected, the new virus should be analysed for virulence and capacity to spread.

3. The notifier and the investigators apply the protocol, the biosafety monitoring and, if necessary, emergency measures as described in the dossier and the accepted amendments.

4. Any protocol amendment, which could have biosafety implications, has to be reported to the competent authority.

The Council wants also to relay the recommendations of its scientific experts in the prospect of a next trial (point 1 set out below) or a future application for a marketing authorisation (points 2 and 3 set out below):

1. If in a next trial the notifier wishes to include cats from individual owners the scientific reason for doing so should be documented.

2. It is assumed that the attenuation is the result of the sole TK deletion. However, it is not known whether the genetic manipulation did not introduce other genetic modifications that could account, at least in part, for the attenuated phenotype of the TK-FeHV-1. Studies on revertants, i.e. TK- FeHV-1 having acquired the TK gene by recombination using the same technique as for the production of the TK- FeHV-1, would allow addressing this point.

3. Considering that the TK deletion is actually identified as the major cause of attenuation, this does not rule out the virulence activity of other loci, which can undergo recombination. Such an exchange of virulence genes could putatively result in an increased virulence of the recipient virus, even if it is a TK mutant. The notifier is invited to consider this point.

p. o.
B. V. Vaerenbergh

Prof. D. Reheul
President of the Biosafety Advisory Council.

Annex : Expertise report. (ref: BAC_2005_GT_261)

Biosafety Advisory Council

Secretariat

O./ref.: WIV-ISP/BAC/2005_GT_261
Email: gmomed@sbb.ihe.be

EXPERTISE REPORT OF THE GROUP OF EXPERTS MANDATED BY THE BIOSAFETY ADVISORY COUNCIL (MAY 27TH, 2004)

July 8th, 2005

Evaluation of the notification B/BE/04/BV1 of the company Pfizer for deliberate release in the environment of genetically modified organisms other than higher plants for research and development according to Part B, Directive 2001/18/EC and the Royal Decision of 18 December 1998

Coordinator's Summary report
Final version
(replaces document BAC_2004_GT_186)

Scientific coordination by the Service of Biosafety and Biotechnology
Secretariat of the Biosafety Advisory Council
Scientific Institute of Public Health

Coordinators: Alan Fauconnier (SPF Santé Publique) and Monika Sormann (Ministerie van de Vlaamse Gemeenschap)
Experts: Bernard Brochier (ISP), Roland Dobbelaer (WIV), Hans Nauwynck (UG), Etienne Thiry (ULg), Thierry Van den Berg (CERVA), Alain Vanderplasschen (Ulg)

INTRODUCTION

Dossier B/BE/04/BV1 concerns a notification of the company Pfizer for deliberate release in the environment of genetically modified organisms other than higher plants (Part B, Directive 2001/18/EC) according to Royal Decision of 18 December 1998.
The notification has been officially acknowledged on 8 September 2004.

The notification concerns a project of a clinical trial in cats entitled: "Evaluation of the safety of Feline Herpes Virus, bivalent deleted live vaccine, administered as intranasal vaccine to cats".

The coordinator assisted by the Secretariat of the Biosafety Council has asked to ten experts among the members of the current list of experts engaged in analysing GMOs for clinical use to review this proposal. Eight experts agreed to participate to this evaluation process. All the documents were sent to these reviewers. They were asked to give their comments on the proposed questionnaire which is structured in a way that it may grossly fit Annex 2 D1 of the European Directive 2001/18/EC and its guidance notes (2002/623/EC) relative to the risk assessment of genetically modified organisms. Two experts were unable to send their answers on time. All the answers received are compiled in the document appended to this report (ref: BAC_2004_GT_169).
The coordinators summarized the different expert's comments and added their personal view in order to build up this summary report. The conclusions are drawn by the coordinator based on the experts' comments.

1. Questions related to the characteristics of the recipient organism

1.1 What are the hazards for the target organism, the human health and the environment related to the non modified parental organism (including pathogenic effects to human and animals)?

Hazards for the target organism
The non-modified parental organism is the feline herpesvirus 1 (FeHV-1), the aetiologic agent of a severe upper respiratory cat disease referred to as feline viral rhinotracheitis. FeHV-1 occurs worldwide and is widespread in cat population. Clinical symptoms include inappetence, depression, fever, sneezing followed by conjunctivitis and nasal discharges. Sometimes ulcers may develop in the mouth, or the cat/kitten may develop pneumonia. The virus may also induce abortion, stillbirth or infertility. The illness seriousness ranges from a benign disease in cats in general good health and conditions to lethality in very young kitten or immunosuppressed animals. As observed with many herpesviruses, FeHV-1 may establish latent infection (up to 80% of all previously infected cats) resulting in carrier state and possibility of reactivation

Hazards for other animal species including human:
FeHV-1 has a narrow host range, both in vitro and in vivo suggesting that it is unlikely that FeHV-1, or derived recombinant strains, could establish cross-species infection. As stated in the dossier, FeHV-1 in vivo host range seems to be restricted to Felidae. However, isolations have been reported in dogs, but these infections did not induce any diseases. FeHV-1 can thus be considered as non-dangerous for other animal species and man. Accordingly, in the EC classification of infective organisms by risks groups (Council Directive 90/676/CEE of 26 November 1990), Feline Herpes virus is classified into group 1. Similarly, this virus has not been classified as potential hazard for human in the Belgian classification (Brussels region IBGE-BIM, Moniteur Belge 26/02/2002).

Environment:
Like most enveloped viruses and other Herpesviruses, FeHV-1 is poorly resistant to external conditions and survives only a few hours in the environment. FeHV-1 will survive in secretions for only up to 18 hours in a damp environment and up to 12 hours in a dry environment and it is unstable as an aerosol. Because of its lipoprotein envelope, it is highly susceptible to the effects of heat, acids and common disinfectants. However, this brief persistence was shown to be long enough for indirect transmission to occur from cats to cats.

2. Questions related to the characteristics of the GMO

2.1. What are the results of the genetic modification in the modified organism?

The genetic modification aims at obtaining two non-virulent FeHV-1strains expressing either the *gag* or the *env* genes of the Feline Immunodeficiency virus (FIV) that can be used together as FIV vaccine in cats. The recombinant viruses were obtained by replacing a 345 base pair fragment of the thymidine kinase gene (TK) by either the *gag* or *env* FIV gene. The deletion of the TK gene is known to induce a drastic attenuation amongst alphaherpesviruses. The inserted sequences include an early promoter, the coding sequence for FIV proteins (either *env* or *gag*) and polyadenylation sequences. The FIV *gag* gene product encodes the precursor protein for the capsid proteins of FIV, whereas the FIV *env* gene product codes for the envelope glycoprotein of FIV.

2.2 Has the genetic modification been sufficiently characterised for the purpose of evaluating the risks (direct, indirect, delayed, immediate, cumulated) to the target organism, the human health and the environment?

Most of the experts consider that the genetic modification has been sufficiently characterised in terms of location, genetic stability notably. One of them further mentions that previous extensive trials with TK-attenuated alphaherpesviruses have demonstrated the safety of these modified viruses. In the present case, no clinical signs suggestive of Feline Herpes Rhinotracheitis were observed in cats vaccinated with the FeHV-1 vector backbone (TK deletion without insert). All the cats tolerated the vaccine, remained active, continued to eat and gain weight and were presented as healthy animals after vaccination.
However, one expert regrets that the genetic stability study achieved through successive backpassages in susceptible cats does not include comparative sequences analysis of the TK and foreign genes before and after backpassages. These data would reinforce the safety evaluation of the vector.
A second expert highlights the absence of data on revertant viruses. It is assumed that the attenuation is the result of the sole TK deletion, but it is not known whether the genetic manipulation did not

introduce other genetic modifications responsible in part for the attenuation character of the TK- FeHV-1. Complementation studies would allow addressing this point (this concern is only related to the target organism).

2.3 Are the steps taken (and assays used with their sensitivity) to detect and eliminate any contaminating viruses (both replication-competent or replication-defective) or other organisms in the cells or serum used for preparation of the virus stock including any contaminants that may have an impact on the risks satisfactory?

Three experts consider that it is indeed the case, in conformity with CFR (*American code of Federal Regulations*) and PhEur (*European Pharmacopoeia*) requirements for cell banks and viral seedlots. In particular, the FIV inserts have been sequenced and are pure from any unknown sequence. The two recombinant vectors have been plaque purified, screened by restriction endonuclease analysis for the correct insertion of the FIV transcription units and the expression of the *gag* and *env* genes has been tested by Western Blot. However, a complete sequence analysis of the region overlapping the TK and foreign genes of the vector is not available. These data would reinforce the safety evaluation of the vector.

Other experts consider that there are insufficient data to answer this question. The full analytical dossier would be needed.

3. Questions related to the risks for the environment

3.1 Likelihood of the GMO to become persistent and invasive in natural habitats under the conditions of the proposed release.

Environment:
Alphaherpesviruses are not suited for long-term survival outside of the host. Since the GMO has no advantage over wild parental FeHV-1 to survive in the environment, this limits its distribution by shedding from oro-nasal-conjunctival secretions or vaccine spill.

Latency:
The inactivation of alphaherpesvirus TK reduces the amount of replication but does not preclude the latency in the trigeminal ganglia. Indeed, it has been demonstrated for several alphaherpesviruses that a TK deleted strain can establish a latent infection and can possibly reactivate. In the present case, a study could show that latent TK negative FeHV-1 (the GMO) could not be reactivated nor re-excreted after glucocorticoid treatment.
One expert considers that this study suggests that the vaccine virus is probably not present under latent form.
Another expert underlines that although some TK- alphaherpesviruses cannot be reactivated from a neuronal latency site, it was shown that at least one alphaherpesvirus, namely bovine herpesvirus 1 (BoHV-1), deleted in the TK gene, was still able to be reactivated from latency and re-excreted in cattle. He emphasises BoHV-1 and FeHV-1 share many pathogenic properties in their respective host species. He concludes by stating that whether or not reactivation is followed by virus re-excretion in field conditions remains to be studied in more details. This opinion is followed by a third expert, which regrets that the researchers did not look for latent virus by PCR in trigeminal ganglia.

Half of the experts consider that the selective disadvantage of the recombinant viruses due to the TK gene deletion reduces drastically the risk of virus propagation in the cat population.

3.2 Are there any selective advantages or disadvantages conferred to the GMO compared to the parental organism?

TK deletion induces a selective disadvantage as demonstrated in the dossier and for several alphaherpesviruses. However, some experts qualify this assertion.

1. The assertions of the applicant, stating that i) the FIV inserted sequences are not involved in any of the pathogenic or harmful properties of the organism and that ii) there is no known tissue tropism associated with the FIV *env* gene, are not true or, at least, too short. Indeed, according to the literature, it is now generally accepted that the envelope gene of FIV contains an important determinant for host cell tropism, such as the V3 domain. Therefore, the applicant should address the possibility to find the vector in the blood lymphocytes of vaccinated cats and its potential risks. In the same way, the use of a strong CMV promoter for the expression of the FIV genes has not been discussed.
2. Acquiring the TK deletion attenuates the GMO. However, it is not known whether genetic manipulations created other genetic modifications able to further attenuate the vaccine virus, as the revertant virus was not studied (complementation studies).

3.3 Immediate and/or delayed adverse effects on the environment and animal health resulting from potential direct or indirect interactions between the GMO and target organism.

The safety profile of the GMO is consistent with other attenuated live intranasal vaccines and the FeHV-1 vector backbone. There is thus no adverse effect to be expected on the environment and animal health.

3.4 Immediate and/or delayed adverse effects on the environment and animal health resulting from the potential direct or indirect interactions between the GMO and non target organisms.

The experts unanimously consider that there is no adverse effect on non-target organisms, since FeHV-1 specifically infects domestic cats and other felids.
Nevertheless, one expert underlines that the host specificity of the vaccine virus has poorly been illustrated. It was only shown that the vaccine virus does not grow in human cell cultures and that intracerebrally and intraperitoneally inoculated mice do not show adverse reactions. It is regretted that the animals were not sacrificed and examined for virus replication.

3.5 Immediate and/or delayed adverse effects on the human health resulting from the potential direct or indirect interactions between the GMO and people working with, coming in contact with or in the vicinity of the treated animal and/or the GMO (workers, owner of the animal, etc...).

No adverse event expected.

3.6 Is there a significant probability that the GMO will spread from the vaccinated cat to other cats, to humans or to the environment?

Spread from cats to cats

The probability that the GMO will spread from the vaccinated cat to other cats has been differently evaluated by the experts, ranging from very unlikely to very probable. But most consider that the spreading will be transient or not be very efficient.

Spread to humans

No spreading to humans is expected, because of the restricted host range of FeHV-1.

Spread to the environment

Considered as either impossible, or possible but very limited because of the instability of the virus vector outside the host organism.

3.7 What is the possibility of the GMO to reconvert to his wild type form and what are the possible consequences for the target organism, the human health and environment?

Herpesviruses are known to be "recombigenic" *in vitro*, with rate of up to 10 to 21 %. *In vivo*, this frequency is unknown. Nevertheless, such a recombination event would induce reversion to the wild type through restoration of the Tk+ status. The result of such an event is stated to be null as it would be an exchange of the inserts and the Tk gene. The formed viral products are expected to be not worse than the original situation.

Two experts consider that the assessment of a possible recombination is not fully satisfactory.

- The risk of recombination is not negligible since the vaccine is administered intranasally, the natural site of FeHV-1. Moreover, the recombination can take place not only in vaccinated animal but also in surrounding animals. Recombination can also take place in both primary infection and during reactivation from latency. The risk of recombination also lies in recombination in other loci than the TK locus. Two types of recombination events could lead to putatively virulent recombinants:
    - Recombination with wildtype FeHV-1 and TK- FeHV-1 in another locus than TK, with exchange of virulence genes from TK- FeHV-1, increasing therefore the virulence of a wildtype FeHV-1.
    - Recombination with wildtype FeHV-1 and TK- FeHV-1 in another locus than TK, with exchange of virulence genes from wildtype FeHV-1, increasing therefore the virulence of the TK- FeHV-1. In this case, a FeHV-1 strain carrying the TK deletion could be, at least partially, virulent.
- There is a small risk that the new recombinant virus, the field strain with *gag*- or *env*-genes at the position of the Tk gene, may spread and be more virulent than the original vaccine virus in cats. It is important not to ignore this risk. For this field trial, it is sufficient to keep the animals and catteries isolated during two weeks after vaccination in order to keep this risk to a minimum.

3.8 Is there any possibility of gene transfer to other micro-organisms and what will be the selective advantages or disadvantages conferred to those resulting micro-organisms? What are the possible consequences for the target organism, the human health and the environment?

The probability is considered as extremely low by all experts.
There is a theoretical possibility of exchange of *gag* or *env* genes from the TK- FeHV-1 to a FIV infecting the same cat. This event requires co-infection which is possible in mononuclear blood cells for both viruses. However, it is difficult to estimate the risk of a recombinant FIV having acquired those genes and it can be most likely considered as negligible.

4. Questions related to the risk assessment of GMO

4.1 How should you describe the magnitude of the as above identified potential risks related to the GMO?

Unanimously considered as low or not significant.

4.2 How should you classify the as above identified potential risks related to the GMO?

Unanimously considered as low or not significant. One expert specifies that both biological agents, FeHV and FIV, are classified into group 1 according to the European Commission classification, since they are unlikely to cause human disease. FeHV has been classified in class risk of level 2 for animal but has not been classified as potential hazard for human in the Belgian classification.

5. Questions related to the monitoring, waste and emergency plans proposed by the applicant

5.1. Does the monitoring plan proposed by the applicant confirms the validity of the hypotheses issued during the risks evaluation concerning the potential adverse effects and does it allow to identify the occurrence of non-anticipated adverse effects ?

One expert considers that the proposed plan is adequate. All other experts found that the plan is not fully adequate for the following reasons:

- The information is not clear. The monitoring plan seems to be mainly based on voluntary reporting by participants.
- Keeping the cats indoor only when unexpected event are detected is not sufficient. Complementary measures should be taken (see under 5.2)

5.2. Which complementary measures could be considered to improve the monitoring plan?

- A clear and detailed monitoring plan lacks in the technical dossier.
- It should be advised to take nasal swabs at 5 and 10 days post intranasal vaccination. When a concurrent infection with a wildtype FeHV occurs, then it should be examined whether recombination occurred. New rFeHV(field)/FIV*gag*/*env* viruses should be analysed on their virulence and capacity to spread.

- To ask the owners keeping their cats inside during 3 weeks after the trial.

See also under 5.8.

5.3 What are the biosafety measures taken to avoid and/or minimise the spread of the GMO beyond the site of release/treated animal?

The experts are divided on this question.

- One expert considers that the properties of the GMO (TK deleted strains) are such that the spreading is minimal (reduced ability to spread within the infected host and between infected and non infected animals).
- Another highlights the disinfection of surroundings of the places where the cats will be vaccinated, according to standard practices. The GMO is sensitive to conventional chemical agents.
- To keep the animals inside during three weeks following vaccination. To avoid any stressing conditions able to induce virus reactivation. There is no measure able to prevent reactivation of the virus during the entire life of the cat. However, an experiment performed by the applicant allows to postulate that reactivation and re-excretion is not likely to occur. The proposed measures could be therefore sufficient (note of the coordinator. this measure is taken for catteries but not foreseen for cats' owners).
- Two experts consider that no measures were taken. One of them suggests isolating vaccinated animals/catteries during a period of 14 days following vaccination.

5.4. Which type of waste could be generated?

Vaccine vials and applicators as well as cat litter, excreted biological fluids and excreta (biological waste) are generated . Only the formers (vials and applicators) were reported as waste taken into account by the applicant. The litter has been addressed in studies of survival in the environment but not as a waste It is not clear if the biological waste is virus positive or not.

5.5. Is the waste treatment proposed by the applicant satisfactory? Which complementary measures could be considered?

The waste treatment is considered adequate for vials and applicators. However, the biological waste (cat litter, excreted biological fluids and excreta) have not been taken into account. More information is needed, such as the exact meaning of "following internal procedures for recombinant products"?

5.6. When the applicant proposes emergency plans, do those plans assure the control of the potential negative effects?

Most experts answer positively to this question. One expert considered that no (convincing?) plan has been proposed.

5.7. Which complementary measures could be considered to improve the emergency plans?

All experts share a similar point of view. Most consider that no complementary measures are necessary. Based on the experience acquired from the use of other TK deleted alphaherpesviruses, one should be convinced that the emergency plan proposed is by far appropriate to the problem.

The expert who regrets the lack of clarity thinks that the Notifier, most probably correctly, assumed that the organism is safe for the environment in general.

5.8. Do the measures to control the risk, as proposed by the applicant, allow to reduce the potential negative effects? Mention, eventually, the level of decrease of the risk and the level of feasibility of the proposed measure. If not, which other measures could be applied and what are the expected effects?

Experts generally agree on the safety of the GMO for the target species, the human health and the environment. Nevertheless, a number of recommendations are proposed:

- The applicant did not mention in the clinical protocol whether treated vet clinic patients (households) must be maintained/isolated or not inside the owner's house during the monitoring period. If vaccinated cats may walk outside, there is a likelihood of contamination of neighbouring cats (shedding/excretion). Therefore, vaccinated animals should be isolated during a period of 2 (or 3) weeks after vaccination.
- Since the vaccine virus will be applied in cats living in an open environment, in cats which may be infected by FeHV-1 at the time of vaccination, in cats which may be in contact with FeHV-1 infected cats during 2 weeks following vaccination, the risk of recombination and latent persistence in field conditions should be properly assessed by the applicant.
- Oropharyngeal swabs should be taken at 0, 5 and 10 days post intranasal vaccination and at regular intervals over a longer period to assess the GMO excretion/shedding in treated cats (cattery animals in particular) and the likelihood of rise of recombinant viruses. When a concurrent infection with a wild FeHV-1 occurs, the possibility of a recombination should be examined. New rFeHV(field)/FIV*gag*/*env* viruses should be analysed on their virulence and capacity to spread.

6. General or additional Comments

According to one expert, the completed EU document (*see technical dossier: information as required by Annex IIIA of Directive 2001/18*) seems to be of rather mediocre quality (e.g. identification techniques only very generally mentioned, not described); maybe this is because this seems to be a classical vaccine target species safety study and the safety of the GMO vaccine strains is largely assumed (probably correctly as demonstrated in the very adequate ERA).

One of the GMO induced the synthesis of a glycoprotein (Env). In terms of safety, it could be interesting to know if this glycoprotein is incorporated in the envelope of the progeny virions. Indeed, in the latter case the transgene could affect the tropism of the virion. This is particularly important here, as the Env glycoprotein has been shown to mediate the binding of the FIV virus. However, the experiments presented in the dossier demonstrate that the GMOs are safe for the target species.

The biosafety assessment of this vaccine must also take into account the existing knowledge on live-attenuated alphaherpesvirus vaccines, either deleted in a non essential gene or not. Such vaccines can be found against BoHV-1 or pseudorabies virus (SuHV-1) with a deletion in the glycoprotein E gene, for example. These widely used vaccines have not been associated yet with the rise of recombinant viruses and the rise of strains exhibiting an increased virulence. It is therefore tempting to extrapolate these field observations to this application. It means that the trial can be conducted provided the

applicant makes a good assessment of the risk of recombination and latent persistence of the TK-FeHV-1 in field conditions and includes in the protocol procedures able to identify these risks with a reasonable probability.

Comment from the coordinator: The function of the *env* has been reported as a capsid protein encoding gene (p. 179). Further on (p. 180), it is stated that no known tissue tropisms has been associated with the FIV *env* gene product. It is true that no known tissue tropisms has been associated with the FIV capsid protein but the *env* gene product is actually an envelope protein, which has been reported as an important tropism factor through its V3 domain (see experts answer to question 3.2). The dossier should be updated accordingly.

CONCLUSION: LIST OF COMPLEMENTARY INFORMATION/RECOMMENDATIONS TO BE ADDRESSED TO THE NOTIFIER

COMPLEMENTARY INFORMATION

1. Sequences:

- A complete sequence analysis of the region overlapping foreign genes and flanking regions, including the insertion site in the vector, should be provided.
- If available, comparative analysis of the TK and foreign genes sequences before and after backpassages in cats (stability study -Ref 9 and 10 of the dossier) should be provided.

Coordinator's comment on additional info received from the Notifier:

In response to a request of complementary information, the Notifier has provided the nucleotide sequences of both regions overlapping either *gag* or *env* genes and their flanking regions, including the insertion site in the vector. Comparative analysis of the TK and foreign genes sequences before and after backpassages in cats is also available, showing the perfect match of TK and foreign genes sequences before and after backpassages in cats. Moreover, the Notifier addressed the stability of the recombinant viruses by providing PCR and Western blots analyses, demonstrating that TK gene deletion and FIV *gag* and *env* gene inserts remained intact, properly located and stable as compared to the rFeHV-FIV*env* and rFeHV-FIV*gag* master seed virus.

-

2. Tropism:

- The function of the env has been reported as a capsid protein encoding gene (p. 179). Further on (p. 180), it is stated that no known tissue tropisms have been associated with the FIV env gene product. It is true that no known tissue tropisms have been associated with the FIV capsid protein but the env gene product is actually an envelope protein, which has been reported as an important tropism factor through its V3 domain. The dossier should be updated accordingly.
- The final localisation of the proteins Env and Gag in the recombinant virion and/or in the infected cells does not clearly appear. The applicant should address the possibility of finding the Env protein in the envelope of the rFeHV-FIVenv virion particles. This point should be considered together with the possibility of finding the vector in the blood lymphocytes of vaccinated cats and its putative risk. In this regard, the use of a strong CMV promoter for the expression of the FIV genes has not been discussed.

Coordinator's comment on additional info received from the Notifier:

In answer to the question, the Notifier specified that direct attempts to detect the presence of the FIV gene product in the FeHV recombinant vector have not been made. However the data to date do not support the presence of a functionally active protein in the recombinant viruses for the following reasons: the Gag protein was never considered a realistic candidate for inclusion into the recombinant herpesvirus, because it is the protein that generates the capsid of FIV and as such is physically too big to be part of the herpesvirus. In addition as both FIV and FeHV are enveloped, the capsid structure is not part of the mechanism involved in targeting and infecting host cells. Consequently, even if it were possible for the Gag protein to become a structural part of a recombinant FeHV it would not be considered to be involved in altering the pathogenesis of the recombinant virus. As for the Env protein, there are several pieces of evidence that demonstrate that a functionally active form of the FIV

envelope glycoprotein is not within the recombinant herpesvirus vector. The first evidence is that the pathogenesis of the recombinants do not differ from that of the TK deleted FeHV. The strongest evidence for the lack of a functional FIV envelope protein within the recombinant herpesvirus is the inability of the FeHV-env to infect primate cells in vitro, whereas wildtype FIV is known to do so. As for the possibility of finding the vector in the blood lymphocytes, the applicant answered that wild type FeHV-1 is known to be sporadically detectable in liver, spleen and PBMC after infection with some isolates, specifying that this is not a consistent finding with FeHV-1 as the primary areas of replication are associated with tissues of the nasal passages and oropharynx.

3. Monitoring plan:

The information on the monitoring plan is rather terse. A detailed monitoring plan should be provided, taking into account the potential shedding, recombination and latency of the recombinant viruses. The applicant should also consider the inclusion of PCR tests for detecting latent virus in trigeminal ganglia.

Coordinator's comment on additional info received from the Notifier:

Shedding – Spread:

Catteries: The Notifier proposes to collect intra nasal swabs every 3 days, from 6 days to 27 days after vaccination for the first 10 cats enrolled in the study. The collected swab will be analysed for virus recovery. In expert's opinion, this proposal must be supported by a rational justification. A longer testing period could be required (see below, under Recommendations).

Owners: As recruiting cats and logistics consequences in the introduction of a new sampling requirements in cats' owners are an issue for such a study, the Notifier believes that there the monitoring plan in catteries should suffice. This position of the company, underlying the difficulty of working with owners' cats, is an additional reason for restricting the clinical trial to cats kept in a controlled environment (catteries, veterinary clinics or practices) (see below, under Recommendations).

Recombination:

According to the Notifier, the results in the backpassage study reports showed that the rFeHV-FIV*env* and rFeHV-FIV*gag* were phenotypically and genotypically stable. The theoretical risk for a co-infection with a wild type FeHV to restore virulence via homologous recombination is unlikely to happen. However, this hypothetic result of such a recombination would be that the wild-type virus gains the ΔTK genotype. In addition, the limited homology between the TK genes of the different members of alphaherpesviridae, the low probability of co-infection of the same cell at the same time of different viruses in stoichiometric amount is extremely low. Therefore, the Notifier believes that a monitoring of a potential recombination is not necessary under field conditions.

According to the experts, this opinion has to be qualified. Indeed, it has been demonstrated that herpesviruses are recombigenic both in vitro and in vivo. Since the vaccine is administered intranasally, the probability of coinfection is not negligible in a same individual already infected with FeHV-1 because the anterior respiratory tract is the natural site of this virus. Therefore, a monitoring of a potential recombination under field conditions makes sense (see below, under Recommendations).

Latency:

The Notifier reminds that studies were conducted on cats treated with glucocorticoid, demonstrating that FeHV vectors included in the vaccine do not recrudesce following administration

of immunosuppressive agents. Therefore, the notified believes that a monitoring of latency is not necessary under field conditions.

This opinion is not totally shared by the experts. They highlight that although some TK- alphaherpesviruses cannot be reactivated from a neuronal latency site, it was shown that at least the bovine herpesvirus 1 (BoHV-1) deleted in the TK gene, was still able to be reactivated by induction with dexamethasone. Spontaneous reactivation has not been shown. It must be emphasized that BoHV-1 and FeHV-1 share many pathogenic properties in their respective host species. The limited experiment carried out by the applicant suggests that the putative TK- FeHV-1 reactivation and re-excretion does not occur or at a low rate. However, it would be advisable to monitor the possibility of reactivation and re-excretion under field conditions over a longer period (see below, under Recommendations).

The possibility of making PCR testing of latent viruses in trigeminal ganglia was raised by one expert in his original assessment and further submitted to the Notifier. The Notifier excludes this possibility since the study is designed to be conducted under field conditions and therefore requires animals from owners. The position of the Notifier is deemed acceptable.

4. Waste:

- It is not clear whether cat litter, excreted biological fluids and excreta of vaccinated animals are virus positive or not. This should be clarified.
- Depending on the answer to this question, the way this waste is treated should be specified.

Coordinator's comment on additional info received from the Notifier:

The Notifier answered as follows: the shedding of vaccinated cats is known to be located in the oropharyngeal sphere. The Notifier is not aware of biological fluids and excreta from vaccinated cats being positive. However, an investigation was carried out on the survival of the vaccine strains in cats' litter tray. Samples of approximately $10^{7.7}$ $TCID_{50}$ of vaccine viruses or wild type FeHV-1 were added to 100 g of kitty liter. After 3 hours, no viruses were detectable. On this basis, the Notifier believes that the waste in cats' litter should not be treated. This answer is deemed acceptable.

5. The applicant did not mention in the clinical protocol whether treated veterinary clinic patients (household pets) must be maintained/isolated or not inside the owner's house during the monitoring period. Clarification is expected.

Coordinator's comment on additional info received from the Notifier:

The Notifier clearly states that in order to mimic what would be the vaccinal scheme under field conditions there is no intention to isolate household cats after vaccination. This is contradictory to the precautionary measures proposed by the experts (see below, under Recommendations).

RECOMMENDATIONS

Experts generally agree on the safety of the GMO for the target species, the human health and the environment. However, a number of precautionary measures should be taken. These measures have been split into requirements that must be fulfilled for conducting the proposed trial and points that should be addressed in the prospect of a future application for a marketing authorisation.

a. Requirements/commitments to be fulfilled:

- If vaccinated cats may walk outside, there is a likelihood of contamination of neighbouring cats (shedding/excretion). Therefore, vaccinated animals should be isolated during a period of 2 (or 3) weeks after vaccination. The compliance of the cat's owners to this requirement raises question.

- Oropharyngeal swabs should be taken over a given period in order to monitor the putative re-excretion of vaccine virus. Swabs positive for the presence of a Herpesvirus should be analysed in order to discriminate putative recombined rFeVH/wildtype isolates from the vaccinal and/or the wildtype parent strains. Since most nasal swabs collected soon after vaccination are expected to be positive for rFeVH*env*/*gag*, not all early swabs must necessarily be tested. The Notifier is thus asked to propose a timetable covering an adequately justified period post vaccination, which includes a molecular testing of given positive samples selected on a rational basis. The number of cats to be tested, the number and schedule of swabs per animal and the extent of the testing period should be based on the expected frequency of wildtype FeHV reactivation, in order to have a reasonable probability of isolating any putative re-excreted Herpesvirus . Moreover, any cat presenting unexpected clinical signs that could be related to a FeHV infection must be swabbed. The resulting sample must be carefully analysed for the presence of vaccinal/wildtype/recombined FeHV strains. Should a recombined isolate be detected, the new virus should be analysed for virulence and capacity to spread.

b. Recommendations in the prospect of a future application for a marketing authorisation

- It is assumed that the attenuation is the result of the sole TK deletion. However, it is not known whether the genetic manipulation did not introduce other genetic modifications that could account, at least in part, for the attenuated phenotype of the TK-FeHV-1. Studies on revertants, i.e. TK- FeHV-1 having acquired the TK gene by recombination using the same technique as for the production of the TK- FeHV-1, would allow addressing this point.

- Considering that the TK deletion is actually identified as the major cause of attenuation, this does not rule out the virulence activity of other loci, which can undergo recombination. Such an exchange of virulence genes could putatively result in an increased virulence of the recipient virus, even if it is a TK mutant. The Notifier is invited to consider this point.

Dr. A. Fauconnier
Coordinator of the Group of experts

Annex: Compilation of all the answers of the experts in charge of evaluating the dossier B/BE/04/BV1 (ref: BAC_2004_GT_169)

Bioveiligheidsraad
Conseil de Biosécurité

September, 29th, 2004

Secretariaat
Secrétariat

O./ref.: WIV-ISP/BAC_2004_GT_169
Email: GMOMED@sbb.ihe.be

Answers of Experts in charge of evaluating the dossier B/BE/04/BV1

Domains of expertise: virology, recombinant herpes virus vectors, design and production of vectors, vaccination of animals, animal health, human health.
Mandate for the Group of Experts: mandate of the Biosafety Advisory Council (BAC) of May 27th, 2004
Coordinators: Alan Fauconnier (SPF Santé Publique) and Monika Sormann (Ministerie van de Vlaamse Gemeenschap)
Experts: Bernard Brochier (ISP), Roland Dobbelaer (WIV), Hans Nauwynck (UG), Etienne Thiry (ULg), Thierry Van den Berg (CERVA), Alain Vanderplasschen (Ulg)
SBB: Myriam Sneyers, Martine Goossens
Source of questions: Annex 2 D1 of the European Directive 2001/18/EC and its guidance notes (2002/623/EC); the final report of the working group 'structure of advice' of the BAC (ref: BAC_2004_WG_069). Only the questions judged pertinent by the coordinator and the SBB have been retained.

INTRODUCTION

Dossier B/BE/04/V1 concerns a notification of the company Pfizer, Animal Health Group, for deliberate release in the environment of genetically modified organisms (GMO) other than higher plants (Part B, Directive 2001/18/EC) according to Royal Decision of 18 December 1998.
The notification has been officially acknowledged on 8 September 2004.

LIST OF QUESTIONS AND ANSWERS

1. Questions related to the characteristics of the recipient organism:

Context: Annex II of the European Directive 2001/18/EC requests information on the recipient or parental organism

Questions:

1.1 What are the hazards for the target organism, the human health and the environment related to the non modified parental organism (including pathogenic effects to human and animals)?

Answer 1: The non-modified parental organism is a Tk- Feline Herpes Virus with a high degree of species-specificity; it has been shown to be non pathogenic for the target species (cats); for humans and for the environment.

Answer 2:

Hazards for the target organism:

The non modified parental organism is the feline herpesvirus type 1 (FHV1), the aetiologic agent of the feline viral rhinotracheitis. The majority of cases of infectious respiratory disease in cats are caused by either FHV1 or feline calicivirus (FCV). **FHV1 occurs worldwide and is widespread in cat populations.** FHV1 infection is generally more widespread in colony animals (boarding catteries, breeding colonies, stray-cat homes,…) compared to isolated household pets**.** FHV1 has one serotype and is unrelated antigenically to herpes viruses tested from other species. FHV1 infection is confined to members of the cat family and is generally a **severe upper respiratory disease** particularly in young, susceptible cats (Crandell et al., 1961). Early signs of the disease include depression, marked sneezing, inappetence and pyrexia, followed rapidly by serous ocular, conjunctivitis and nasal discharges. Dyspnoea and coughing may develop. Other signs seen less commonly include tongue ulcers, ulcerative keratitis and a primary viral pneumonia. Generalized disease may also occasionally occur, particularly in younger or immunosuppressed animals (Spradbrow et al., 1971; Shields & Gaskin, 1977; Van Pelt & Crandell, 1987). It is probable that abortion, sometimes associated with the respiratory form of FHV1 infection, are secondary to the severe debilitating respiratory infection rather than a direct effect of the virus itself (Hoover & Griesemer, 1971). The mortality rate is not usually high, except sometimes in very young kittens or immunosupressed animals. Signs generally resolve within 10-20 days.

Hazards for the human health and the Environment:

None. FHV1 is confined to felid cells and infection to members of the cat family (Yokoyama et al., 1995; Rota et al., 1986; Martin et al, 2003)**.**

The FHV1 persistence in the external environment, although this is for a only relatively short periods of time is a way among others of persistence of FHV1 in cat populations. This persistence of FHV1 in the external environment is long enough for indirect transmission to occur from cats to cats.

FHV1 will survive in secretions for only up to 18 hours in a damp environment and up to 12 hours in a dry environment (Povey & Johnson, 1970) and it is unstable as an aerosol (Donaldson & Ferris, 1976). Because of its lipoprotein envelope, it is highly susceptible to the effects of heat, acids and common disinfectants (Scott, 1980).

Answer 3: Feline herpesvirus type 1 (FHV) has a specific tropism for cats (domestic and wild). Therefore, FHV can be considered as not dangerous for other animal species and man.

Answer 4:

Hazards for the target organism:

Both recombinant strains produced are deleted for the TK gene. The effect of TK inactivation has been studied extensively for a large number of alphaherpesvirnae. For all viruses tested, the deletion of the TK gene has led to the attenuation of the virus. The results presented in the dossier demonstrated that this is also the case for Feline herpesvirus 1 (FeHV-1). In conclusion, the two recombinant strains produced do not represent a danger for the target species.

Hazards for other animal species including human:

FeHV-1 has a narrow host range, both in vitro and in vivo suggesting that it is unlikely that FeHV-1, or derived recombinant strains, could establish cross-species infection. As state in the dossier, FeHV-1 in vivo host range seems to be restricted to Felidae. However, some isolations have been reported in dogs, but these infections did not induce any diseases (Rota et al., 1986, Kramer et al., 1991).

Human cells are resistant to FeHV-1 infection. The resistance is due to a blockage at the entry stage. This feature further supports the safety of FeHV-1 recombinants for humans (Tegtmeyer and Enders, 1969).

Answer 5 :

- Target organism (cat): Feline Herpes Virus (FHV) is a causative agent of cat flu, a benign disease in cat in general good health and conditions. Clinical symptoms include fever, sneezing, conjunctivitis, depression and inappetence. Most cats recover in 1 to 2 days. The virus can sometimes induce abortion, stillbirth or infertility. FHV may also establish latent infection resulting in carrier state and possibility of reactivation. (Tegtmeyer & Enders, 1994; Maeda et al., 1998). The virus is classified in class risk of level 2 for animal in the Belgian classification (Brussels region IBGE-BIM, Moniteur Belge 26/02/2002).

- Human health: according to the EC classification of infective organisms by risks groups (Council Directive 90/676/CEE of 26 November 1990), Feline Herpes virus is classified into group 1, since this is a biological agent unlikely to cause human disease. There is no evidence of the ability of FHV to replicate or cause disease in human. Moreover, the parent virus is unable to replicate in non-felid culture cells (ref 60 of the dossier). This virus has not been classified as potential hazard for human in the Belgian classification (Brussels region IBGE-BIM, Moniteur Belge 26/02/2002).

- Environment: Like most enveloped viruses and other Herpesviruses, FHV is poorly resistant to external conditions and survives only a few hours in the environment. Follow-up of vaccinated cats (ref 55, 56 of the dossier) as well as sentinel cats (ref 3, 4 of the dossier) demonstrated that there is viral shedding of low levels of vaccine during about 2 weeks after inoculation. This represents the only way of dissemination of the virus. This limited horizontal transmission was ineffective in inducing any clinical symptoms in sentinel cats, even when those were neonatal kittens or pregnant cats.

Answer 6 : Non pathogenic effect on human, because feline herpesvirus 1 (FeHV-1) is an exclusive animal pathogen, infecting domestic cat, cheetah, puma and lion. One observation has been also made in dog (Thiry, 2002).

2. Questions related to the characteristics of the GMO:

Context: Annex II of the European Directive 2001/18/EC requests information on the result of the genetic modification in the modified organism (for example: number of copies of the transgenes, stability of the transgenes, modification in the expression of the genes, re-arrangements in the genome, inclusion or suppression of genetic material)

Questions:

2.1. What are the results of the genetic modification in the modified organism?

Answer 1: Feline Immunodeficiency Virus sequences (env or gag) have been inserted at the site of the Tk deletion of the FHV vector.

Answer 2:

The objective of the genetic modification is to obtain a non-virulent FHV1 expressing the two proteins of the Feline Immunodefficiency virus (FIV), that can be used as a vaccine in cats.

Deletion of genetic material

FHV1 is attenuated through a 345 base pair insertional deletion that was engineered into its thymidine kinase gene (TK) whose the function is essential for normal virus multiplication and pathogenicity in permissive hosts. **The TK gene inactivation through deletion is recognized as a way to attenuate alphaherpesviruses** (e.g. pseudorabies virus, bovine herpesvirus, herpes simplex virus)(Kit et al., 1985; Field & Wildy, 1978; Marchiolly et al., 1987; Yokoyama et al., 1996).

Insertion of genetic material

A transcription unit is inserted in the TK deleted locus of FHV1. The transcription unit contains an early promoter, the coding sequence for FIV proteins (either env or gag) and polyadenylation sequences. The FIV gag gene products encodes the precursor protein for the capsids proteins of FIV. The FIV env gene product encodes for the envelope glycoprotein of FIV.

Answer 3: The deletion of the Tkgene results in an attenuation of FHV. This is a general feature for most alphaherpesviruses. At this position the gag- or env- gene of FIV has been introduced.

Answer 4: The recombinant strains produced are TK negative as the result of the deletion/insertion process performed. As mentioned above, TK deletion in various *alphaherpesvirinae* has been demonstrated to induce a drastic attenuation.

Answer 5: The objective of the genetic modification was to obtain a non-virulent FHV virus expressing two proteins of FIV (gag & env) that can be used as FIV vaccine in cats. The vaccine is comprised of two FHV vectors. The biological agents were made by inserting a cassette encoding the genes of FIV into the thymidine kinase (TK) gene of a FHV by homologous recombination. The resulting FHV vector was attenuated due to the deletion in the TK locus (TK-). No clinical signs suggestive of Feline Herpes Rhinotracheitis were observed in cats vaccinated with the FHV vector backbone (TK deletion without insert). All the cats

tolerated the vaccine, remained active, continued to eat and gain weight and were presented as healthy animals after vaccination. (Yokoyama et al., 1996 + references nr 2, 12, 13 and 15 to 30 of the dossier).

Answer 6: Numerous papers report that the deletion in the thymidine kinase gene results in an attenuation in alphaherpesviruses (Gogev et al., 2003). This observation is made also with the GMO under concern.

2.2 Has the genetic modification been sufficiently characterised for the purpose of evaluating the risks (direct, indirect, delayed, immediate, cumulated) to the target organism, the human health and the environment?

Answer 1: Yes

Answer 2: The genetic modification has been sufficiently characterised *(See 2.1)*.

Answer 3: Yes.

Answer 4: Yes. Moreover, previous extensive trials with TK- attenuated alphaherpesviruses (PrV for example) have demonstrated the safety of these modified viruses.

Answer 5: The vectors have been plaque purified and molecularly characterized (PCR, restriction analysis & Western Blotting). To confirm their genetic stability, both rFHV-FIVenv and rFHV-FIVgag were passaged sequentially 5 times in cats. Viruses recovered from oro-pharyngeal tissues after five passages were analysed by PCR and compared with the original inocula. The PCR and Western blot data indicated that the gene inserts remained intact, properly located and stable as compared to the material prior to passages in cats. These data support the conclusion that the virulence-attenuating gene deletions in both vaccine components are genetically stable. However, comparative sequences analysis of the TK and foreign genes before and after passages are not available. These data would reinforce the safety evaluation of the vector (Ref 9 and 10 of the dossier).

Answer 6:

- The Company does not report the production of the revertant virus, i.e. FeHV-1 previously deleted in thymidine kinase (TK) gene and having acquired either feline immunodeficiency virus (FIV) gag or env, and experimentally rescued by genetic recombination with a plasmid carrying the entire FeHV-1 TK gene. The properties of such revertant should be explored, in order to ensure that it exhibits the virulence properties of the parental strain, i.e. wildtype UT88-1729, isolated from a clinical case. It is indeed assumed that the attenuation is carried only by the TK deletion, but it is not known whether the genetic manipulations did not introduce other genetic modifications responsible in part for the attenuation character of the TK negative (TK-) FeHV-1.
- This has only implications for the target organism.

2.3 Are the steps taken (and assays used with their sensitivity) to detect and eliminate any contaminating viruses (both replication-competent or replication-defective) or other organisms in the cells or serum used for preparation of the virus stock including any contaminants that may have an impact on the risks satisfactory?

Answer 1: Yes in conformity with CFR (*American code of Federal Regulations*) and PhEur (*European Pharmacopoeia*) requirements for cell banks and viral seedlots.

Answer 2: Is not documented.

Answer 3: Yes.

Answer 4: The FIV inserts have been sequenced and are pure from any unknown sequence. The two recombinant vectors have been plaque purified, screened by restriction endonuclease for the correct insertion of the FIV transcription units and the expression of the *gag* and *env* genes have been tested by Western Blot. However, once again, complete sequence analysis of the region overlapping the TK and foreign genes of the vector is not available. These data would reinforce the safety evaluation of the vector.

Answer 5: Insufficient data in the providing files to answer this question. Need for the full analytical dossier.

3. Questions related to the risks for the environment:

Context: Annex II D.1 of the European Directive 2001/18/EC requests information on the environmental impacts resulting from direct and indirect interactions between the GMO and other organisms.

Questions

3.1 Likelihood of the GMO to become persistent and invasive in natural habitats under the conditions of the proposed release.

Answer 1: Unlikely as documented in the ERA (*Environmental risk assessment*) report.

Answer 2:

The GMO as well as FHV1 may initially be found in oro-nasal-conjunctival secretions (Gaskell & Povey, 1979). Exposure can occur from these sources, as well as from a vaccine spill. In the case of a spill, alphaherpesviruses are not particularly suited to long term survival outside of the host (*see 1.1*)(Turner et al, 1982) and the genetic manipulation made to the FHV1 backbone would not provide any physical modifications to the viruses that would increase their durability or survivability in the environment. **The GMO has no advantage over wild parental FHV 1 to survive in the environment, thus limiting distribution (*see 1.1*)**.

As a typical alphaherpesvirus, the GMO can theoretically persist in the vaccinated/infected cat as a carrier state. The carrier state is the normal sequel to FHV 1 infection and an important way for FHV 1 to persist in cat populations. It is characterized by periods of latency interspersed with intermittent episodes of virus shedding, particularly after a stress (change of housing, entering a boarding cattery, lactation,…). During shedding episodes, virus can be detected in

oronasal or cunjunctival secretions and the cats are infectious to other cats (Gaskell & Povey, 1982). Although over 80% of FHV 1-recovered cats remain as virus carriers, approximately only half of these are likely to be of epidemiological importance, i.e. likely to shed virus under natural conditions. The trigeminal ganglia is the reported site of FHV1 latency (Gaskell et al., 1985).

The inactivation of alphaherpesvirus TK reduces the amount of replication but doesn't preclude the latency in the trigeminal ganglia. However a study could show that latent TK negative FHV1 (the GMO) could not be reactivated nor re-excreted through the use of immunosupressants (Berlinski et al., 2003).

Answer 3: Latency is important for FHV to persist in cat populations. After primo-infection, virulent FHV stays in its host under a latent form in the ganglion trigeminale. With the vaccine rFHV-FIVenv/gag, it was not possible to reactivate the virus in vaccinated animals. This shows that the vaccine virus is probably not present under latent form. It is a pity that the researchers did not look for latent virus by PCR, which is a very sensitive method. FHV may also persist by continuous circulation (primo-infections and re-infections). With the present vaccine virus, it was shown that the virus can be transmitted, but in a very restricted way. This means that the vaccine virus will not cause a chain of virus transmissions in between animals like the virulent parental strain. In conclusion, we expect that the vaccine virus will not persist in a population.

Answer 4: It has been demonstrated for several alphaherpesviruses that a TK deleted strain can establish a latent infection and eventually be reactivated (Mengeling, W. L., 1991, Whetstone, C. A. et al, 1992). Consequently, vaccinated animals will probably represent reservoirs of GMOs and could possibly lead to virus excretion long after vaccination. However, the selective disadvantage of the recombinant viruses due to the TK gene deletion reduces drastically the risk that the virus could propagate in the cat population. The latter conclusion is also supported by some of the experiments presented in the dossier.

Answer 5: The deletion of the FHV TK alters the pathogenesis of the virus in the cat. The modification has been shown to reduce the replication (duration & amplitude) in the primary target tissues (oral, pharyngeal and saliva). Vectors included in the vaccine have not demonstrated any altered tropism as compared with wild type. Efforts to recrudesce latent vectors through the use of immunosuppressants have been unsuccessful.

Answer 6:

FeHV-1 is a common pathogen of cat. It is excreted by the nasal and ocular secretions over about two weeks after a primary infection (Thiry, 2002). FeHV-1 is an alphaherpesvirus and persists in a latent state in cat. It can be reactivated either by glucocorticoid treatment or by diverse natural stimuli. FeHV-1 is therefore easily transmitted to other cats in the conditions of primary infection or during re-excretion after reactivation from latency (Thiry, 2002).

The deletion in the TK gene is usually associated with a loss of infectivity and a decreased permissivity in neurons (Gogev et al., 2003). Although some TK- alphaherpesviruses cannot be reactivated from a neuronal latency site, it was shown that at least one alphaherpesvirus, namely bovine herpesvirus 1 (BoHV-1), deleted in the TK gene, was still able to be reactivated from latency and re-excreted in cattle (Whetstone et al., 1992). It must be emphasized that BoHV-1 and FeHV-1 share many pathogenic properties in their respective host species. A limited experiment carried out by the applicant did not show reactivation and re-excretion in 10 cats

infected with a TK- FeHV-1 and submitted to a glucocorticoid treatment 10 weeks after infection. It may be therefore postulated that the potential of TK- FeHV-1 reactivation and re-excretion is low. Whether or not reactivation is followed by virus re-excretion in field conditions remains to be studied in more details.
These data are required for the assessment of the persistence of the vaccine strain in the cat population.

3.2 Are there any selective advantages or disadvantages conferred to the GMO compared to the parental organism?

Answer 1: None known (ERA report)

Answer 2: The inactivation of alphaherpesvirus TK reduces the amount of replication but doesn't preclude the latency in the trigeminal ganglia. However a study could show that latent TK negative FHV (the GMO) could not be reactivated nor re-excreted through the use of immunosuppressants.

Answer 3: No (see 3.1.).

Answer 4: TK deletion induces a selective disadvantage as demonstrated in the dossier and for several alphaherpesviruses.

Answer 5:

On one hand, considering the recipient organism, the GMO is attenuated as compared with the wild type. This can be considered as a selective disadvantage.
On the other hand, considering the donor organism, there is a lack of pertinent information in the dossier regarding the risk of potentialisation of the vector by the FIV inserts. In the Summary Notification, point C, 3 (b): To the question: "Are the donated sequences involved in any to the pathogenic or harmful properties of the organism", the applicant answer is "no" and this is justified as "In cats, the FIV env protein elicits an immune response to FIV. There are no known tissue tropism associated with the FIV env gene". This is not true or, at least, too short. Indeed, according to the literature, it is now generally accepted that the envelope gene of FIV contains the determinant for host cell tropism. FIV demonstrates a broad tropism for peripheral blood mononuclear cells: while CD4+ cells did carry the highest burden of virus during the acute stage of infection, Ig+ cells carried a greater burden of virus in the chronically infected cats (Tompkins et al., 1993, Journal of Virology 67 (9): 5175-86.). Feline CD9 has been proposed as one of the receptor of FIV, analogous to the HIV receptor CD4 (Neil et al., 1994, Immunology, 81(2): 228-33). Investigating the issue of tropism, which is crucial for pathogenicity, researchers found it necessary to look for a tropism segment of the genome. In fact, the FIV tropism has been successfully mapped to the 3' end of the FIV genome, and it has been proven that a single amino acid mutation in the V3 variable region of the viral envelope is directly responsible for the variation in cell tropism between two variants of the virus (Verschoor et al., 1995; Dean et al., 1999). Interestingly, this region of the genome (the V3 region) is also an important neutralization domain. The fact that a tropism determinant and a neutralization domain are present in the same genomic region suggests that V3 is necessary for the entry of FIV into lymphoid cells. The mechanism by which V3 interacts with surface membrane proteins is however, unknown at this time.

This important question needs to be addressed by the applicant. Has the possibility to find the vector in the blood lymphocytes of vaccinated cats been investigated? Which are the potential risks?

In the same way, the use of a strong CMV promoter for the expression of the FIV genes has not been discussed.

Answer 6: The GMO is attenuated by acquiring the TK deletion. It is not known whether genetic manipulations created other genetic modifications able to further attenuate the vaccine virus, as the revertant virus was not studied.

3.3 Immediate and/or delayed adverse effects on the environment and animal health resulting from potential direct or indirect interactions between the GMO and target organism.

Answer 1: None known (ERA report)

Answer 2: Clinical observations of occasional sneezing or mild, transient serous nasal or ocular discharges were observed in animals given the GMO vaccine intranasally. Clinical signs suggestive of feline viral rhinotracheitis were not observed in any of the vaccinated cats. The transient discharges were attributed to the initial replication of the attenuated herpesviruses in the nasal and ocular epithelial tissues (Berlinski et al., 2003). This safety profile is consistent with other modified live intranasal vaccines and the FHV1 vector backbone (TK deletion without the FIV gene inserts) (Wardley et al., 1992; Guimond et al., 1993).

Answer 3: Cats, intranasally vaccinated with rFHV-FIVenv/gag may show transient clinical signs, consisting of sneezing, nasal and ocular serous discharge. In general, fever does not occur. Especially young animals of < 3-4 weeks are susceptible. At this age, almost all animals are showing clinical signs. In older animals, only 10% of the cats have problems. Since young cats will be excluded from this trial, we expect an allowable vaccination reaction in a restricted number of animals. We do not expect adverse effects on the environment.

Answer 4: Not probable

Answer 5:

There are no adverse effect to be expected on the environment and animal health. The vaccine could only replace the wild type virus on the long-term, what should be beneficial for both the environment (attenuated strain) and the animal (protection against FHV & FIV).

Several trials have been conducted by the manufacturer in cats ranging from 7 to 12 weeks of age at the time of vaccination. Clinical signs suggestive of feline viral rhinotracheitis were not observed in any of the vaccinated animals.

Answer 6: No expected interactions between the GMO and target organism. The consequences of recombination are evaluated under point 3.7.

3.4 Immediate and/or delayed adverse effects on the environment and animal health resulting from the potential direct or indirect interactions between the GMO and non target organisms.

Answer 1: None known (ERA report)

Answer 2: None adverse effect. Both FHV1 (parental organism) and GMO are confined to felid cells (Yokoyama et al., 1995; Rota et al., 1986; Martin et al, 2003).

Answer 3: The host specificity of the vaccine virus has poorly been illustrated. It was only shown that the vaccine virus does not grow in human cell cultures and that intracerebrally and intraperitoneally inoculated mice do not show adverse reactions. It is a pity that the animals were not euthanatized and examined for virus replication. However, we do not expect changes in host-specificity of FHV by deletion of the TK gene and introduction of the env-gene and the gag-gene of FIV.

Answer 4: Not probable

Answer 5: None.

Answer 6: No adverse effect, since FeHV-1 infects domestic cats and other felids.

3.5 Immediate and/or delayed adverse effects on the human health resulting from the potential direct or indirect interactions between the GMO and people working with, coming in contact with or in the vicinity of the treated animal and/or the GMO (workers, owner of the animal, etc...).

Answer 1: None known (ERA report)

Answer 2: None adverse effect. Both FHV1 (parental organism) and GMO are confined to felid cells (Yokoyama et al., 1995; Rota et al., 1986; Martin et al, 2003).

Answer 3: None

Answer 4: Not probable

Answer 5: None.

Answer 6: No adverse effect, since FeHV-1 is known to only infect domestic cats and other felids (Thiry, 2002).

3.6. Is there a significant probability that the GMO will spread from the vaccinated cat to other cats, to humans or to the environment?

Answer 1: Likely to other cats but not persistent; exposure of humans and environment possible but no infection demonstrated.

Answer 2: As the GMO is confined to felid cells and can be excreted/shedded by freshly vaccinated cats, there is a significant probability of transmission of low virus titers from vaccinated cats to other cats (see 3.1) but not to humans nor to the environment.

Answer 3: It has been shown by the producer that the vaccine spreads from intranasally vaccinated cats to unvaccinated, in contact animals. However the spread was not very efficient. Several in contact animals remained uninfected. This means that the vaccine virus is strongly handicapped in its ability to spread.

Answer 4: Based on the arguments presented above (please see points 1.1 and 3.1), it is very unlikely that the virus will spread from vaccinated cats to other cats and it is impossible that vaccinated cats could lead to human infection by the GMOs.

Answer 5:

- Spread from vaccinated cats to other cats: Yes, but only during a short period of time not exceeding 2 weeks.

- Spread to humans: No. The host range of FHV is very restricted and only affects cats. FHV does not replicate in non-felid mammals (e.g. experimental intracerebral inoculation of mice, ref 11 of the dossier) and several mammalian cell models used (ref. 61 of the dossier). In addition, data suggest that carriage and shedding of the vaccine virus are both limited and transient in fully permissive host.

- Spread to environment: Possible but limited. Like other alphaherpesviruses, the virus is poorly resistant in the environment (ref 62 to 66 of the dossier). Experimental simulations performed with a bench spill test, kitty litter or water indicated a similar rate of decrease between vaccine and wild type virus. On the other hand, viral shedding is transient and does not occur in appreciable amounts. This limited bioburden is unstable in the normal feline environment. Horizontal transmission by vaccinated cats is also very limited by their social behaviour and limited direct contact with non-target animals. The probability to colonize alternate household pets, rodents, livestocks or wildlife is therefore extremely low. This is comforted by experimental inoculation of rodents and dogs that failed to replicate or cause disease in non-felid species (ref. 44 and 55 to 59 of the dossier).

Answer 6:

There is a high probability that the GMO spreads from the vaccinated cat to other cats (see this application), but not to humans and not to the environment.

FeHV-1 is a common pathogen of cat. It is excreted by the nasal and ocular secretions over about two weeks after a primary infection (Thiry, 2002). FeHV-1 is an alphaherpesvirus and persists in a latent state in cat. It can be reactivated either by glucocorticoid treatment or by diverse natural stimuli (Thiry, 2002). FeHV-1 is therefore easily transmitted to other cats in the conditions of primary infection or during re-excretion after reactivation from latency.

In this application, the TK- FeHV-1 was shown to be easily transmitted to in contact cats. This is not surprising because the vaccine is administered intranasally, the natural route of infection, and because virus excretion lasts at least 5 days after infection.

3.7 What is the possibility of the GMO to reconvert to his wild type form and what are the possible consequences for the target organism, the human health and environment?

Answer 1: Herpesviruses are stated to be “recombigenic” and reversal to wild type through restoration of the Tk+ status at co-infection is stated to be possible. The result of such an event is stated to be null as it would be an exchange of the inserts and the Tk gene.

Answer 2: *(see 3.8)*

Answer 3: It is impossible to reconvert to his wild type form except upon recombination during a simultaneous vaccination and infection. The possibility of alphaherpesviruses to recombine has clearly been demonstrated. Recently, beautiful work has been done by Thiry and coworkers with the bovine alphaherpesvirus BHV1. They have shown that recombination is a common feature with BHV1 both in vitro and in vivo (Schynts et al., Journal of Virology 77, 12535-12542). When the rFHV-FIVenv/gag would be inoculated intranasally in acutely FHV-infected cats, then recombination may be expected. The result will be: restoration of the vaccine virus with the formation of the wild type form and the formation of a field strain in which the TK gene is replaced by the env- or gag- gene. The formed viral products are expected to be not worse than the original situation. However there may be one small risk. It is possible that the new recombinant virus, the field strain with gag- or env-genes at the position of the Tkgene, may spread and be more virulent than the original vaccine virus in cats. It is important not to ignore this risk. For this field trial, it is sufficient to keep the animals and catteries isolated during two weeks after vaccination in order to keep this risk to a minimum.

Answer 4: As stated in the dossier, the only possible scenario for the GMO to reconvert to the wild type genotype is to recombine with a wild type strain. Based on this process of homologous recombination, the GMO will become a wild type strain and the donor wild type strain will become a GMO. In other words, this putative recombination process won't have any effect.

Answer 5: see 3.8

Answer 6:

Consequences for the target organism, not for the human health, not for the environment.
The assessment of recombination is not fully satisfactory.

- FeHV-1 easily recombines in every segment of its genome, in a situation of co-infection. This phenomenon is shared by most alphaherpesviruses (Thiry et al., 2004). The rate of recombinant viruses *in vitro* is 10 to 21 % (Fujita et al., 1998). *In vivo*, this frequency is unknown. Based on a comparative work carried out on BoHV-1, an alphaherpesvirus sharing the same pathogenic properties as FeHV-1, *in vivo* recombination may occur at similar rates as *in vitro* (Schynts et al., 2003) provided co-infection or only a slightly delayed superinfection of the same cell occurs (Meurens et al., 2004b). Furthermore, *in vitro*, homologous recombination event occurs between genomically slightly distant alphaherpesviruses (Meurens et al., 2004a; Thiry et al., 2004).
- The vaccine is administered intranasally. Therefore the chance of coinfection is not negligible in a same individual already infected with FeHV-1 because the anterior respiratory tract is the natural site of FeHV-1 replication (Thiry, 2002).
- By analogy with BoHV-1 (Schynts et al., 2003), a high rate of FeHV-1 recombinants can be expected in favourable epidemiological conditions, because FeHV-1 is a common pathogen in cat .
- The recombination event can take place either in the vaccinated individual or a surrounding animal because the vaccine strain is transmitted to in contact cats (see this application).
- The risk of recombination is not linked to the transfer of the deleted TK gene with gag or env gene insertion to a FeHV-1 strain. Indeed the created TK- virus and the expression of gag or env are not likely associated with increased virulence (see this application).
- The assessment of recombination can be made both during primary infection and during re-excretion after reactivation from latency (Dispas et al., 2003).

- The risk of recombination lies also in recombination in other loci than the TK locus:
    - TK- FeHV-1 originated from a wildtype FeHV-1, UT88-1729, isolated from a clinical case. The attenuation was obtained by the TK deletion.
    - There is no information that a revertant virus has a restored virulence. By revertant virus, it must be understood a TK- FeHV-1 having acquired the TK gene by recombination using the same technique as for the production of the TK- FeHV-1. If it is assumed that attenuation is carried exclusively by the TK deletion, the revertant should be fully virulent, because the virulence genes other than TK gene are retained in the TK- FeHV-1. This information is lacking in the dossier.
- The risk of the following situations should be therefore assessed, provided TK deletion is the only attenuation in the genome:
    - Recombination with wildtype FeHV-1 and TK- FeHV-1 in another locus than TK, with exchange of virulence genes from TK- FeHV-1, increasing therefore the virulence of a wildtype FeHV-1.
    - Recombination with wildtype FeHV-1 and TK- FeHV-1 in another locus than TK, with exchange of virulence genes from wildtype FeHV-1, increasing therefore the virulence of the TK- FeHV-1. In this case, a FeHV-1 strain carrying the TK deletion could be, at least partially, virulent.

3.8. Is there any possibility of gene transfer to other micro-organisms and what will be the selective advantages or disadvantages conferred to those resulting micro-organisms? What are the possible consequences for the target organism, the human health and the environment?

Answer 1: None known (ERA report)

Answer 2:

To confirm the genetic stability, the GMO was passaged sequentially five times in cats. The virulence attenuating TK gene deletion in the GMO components were shown to be genetically stable (Berlinski et al, 2003)

There are no known mechanisms for spontaneous generation of the critical 345 bp sequence within the genome such as the spontaneous reversion of a point mutation or a single base deletion.

There is a lack of homology between herpesvirus TK genes and host cellular TK genes and there is limited homology of FHV1 TK with other known herpesvirus TK genes (Yokoyama et al., 1995). That makes unlikely that the FHV1 TK gene can be restored by other herpesviruses or cellular TK genes. However, it is possible for the TK gene to be repaired by homologous recombination with a wild FHV1 containing a functional TK. While little evidence has been documented for recombination between FHV1 isolates in vivo, there is a description of this recombination process in vitro if different viruses were used to co-infect the same cells (Fugita et al., 1998).

This means that the primary method of restoring the virulent TK phenotype would be the unlikely co-infection of the same cell with an intact, TK-positive FHV1 and the GMO vaccine. In this hypothetical case, homologous recombination may occur to restore the TK-positive phenotype of the vaccine strain. However, in doing so, this would generate a TK-negative phenotype in the wild-type donor. The net result would be silent, as the unlikely restoration of the TK-positive phenotype (widespread in the cat population) would result in a reciprocal TK-

negative phenotype due to a virus with the TK deletion containing the FIV antigen expression cassette.

The likelihood of the GMO to reconvert to his wild type form is very low and if so it would have no consequence for the target organism, the human health and the environment.

Answer 3: See 3.7. recombination. No danger for non-feline animals and man.

Answer 4: Homologous recombination requires qualitatively and quantitatively large homologous regions. As no viruses closely related phylogenetically to FeHV-1 have been described, the probability of recombination between the GMOs and wild type strains of an other viral species is very unlikely. However, if such recombination event should occur, it will lead to the production of an attenuated recombinant virus due to the TK deletion resulting from the recombination process; and consequently, to the attenuation of the recipient virus. The probability of homologous recombination between the GMOs and biological entities other than alphaherpesviruses are negligible.

Answer 5:

These two questions (3.7 and 3.8) are closely related as reversion to wild type could only occur through a recombination event. This probability is very low as recombination in vitro is performed under selection pressure, which does not exist under natural conditions, in order to obtain TK- viruses. In addition, the use of the same deletion in both vaccine components prevents the occurrence of recombination with the other vaccine component such as the TK gene would be functionally restored. Finally, there is little homology between FHV TK and other known herpes viruses TK genes (max. 54%) or cellular TK genes (less than 40%). A recombination event will therefore need to have cats infected simultaneously with high doses of wild type and vectors to allow multiplication and recombination in the same cell. In case of recombination (extremely rare and unlikely event), the vector can only revert to the wild type, which is widespread in cat populations.

In addition, interference of replication is a common immune mechanism described in the protection against herpesviruses. This property, based on the observation that cells infected with one herpesvirus are refractory to a surinfection by another one, is used for vaccination purposes (i.e. Marek's Disease).

For all these reasons, reversion or recombination are very unlikely events in the present case (Ref: 33 to 44 in the dossier).

Anyway, it has not been investigated whether the vaccine is able to establish a latent infection in the trigeminal ganglia as well, like its wild-type counterpart. This is highly probable as literature reports that TK- alphaherpesviruses can enter the trigeminal ganglion and become latent (ref 52 & 53 of the dossier). A reactivation event, which has never been reported for TK- viruses, could then represent an exceptional circumstance for recombination, provided that the animal was latently infected with both the wild type and the vaccine. Even in this case, recombination can only give birth to a wild type virus.

Answer 6: There is a theoretical possibility of exchange of gag or env genes from the TK- FeHV-1 to a FIV infecting the same cat. This event requires co-infection which is possible in mononuclear blood cells for both viruses (Thiry, 2002). However, it is difficult to estimate the risk of a recombinant FIV having acquired those genes and it can be most likely considered as negligible.

4. Questions related to the risk assessment of GMO:

Context: The European Directive 2001/18/EC forms the framework for the risk assessment of the deliberate release of GMOs.

Questions:

4.1. How should you describe the magnitude of the as above identified potential risks related to the GMO?

Answer 1: Not significant

Answer 2: *(see 4.2)*

Answer 3: small

Answer 4: Extremely low

Answer 5: Low. This is confirmed by the manufacturer using the methods described in the Risk Characterization section of the APHIS guideline.

Answer 6: Low

4.2. How should you classify the as above identified potential risks related to the GMO?

Answer 1: Not significant

Answer 2: The overall risk rating for the GMO is **very low** and there should be little concern associated with its release into the environment.

Answer 3: Extremely low

Answer 4: Very unlikely

Answer 5: Both biological agents (FHV & FIV) are classified in group 1 according to the European Commission classification (Council Directive 90/679/EEC of 26 November 1990), since they are unlikely to cause human disease. FHV has been classified in class risk of level 2 for animal but has not been classified as potential hazard for human in the Belgian classification (Brussels region IBGE-BIM, Moniteur Belge 26/02/2002). I agree with these classifications.

Answer 6: Low

5. Questions related to the monitoring, waste and emergency plans proposed by the applicant:

Context: The European Directive 2001/18/EC requests from the applicant to propose monitoring, control, waste treatment and emergency response plans

Questions:

5.1. Does the monitoring plan proposed by the applicant confirm the validity of the hypothesises issued during the risks evaluation concerning the potential adverse effects and does it allow to identify the occurrence of non-anticipated adverse effects ?

Answer 1: Not clear to me, seems to be mainly based on voluntary reporting by participants (*see technical dossier: information as required by Annex IIIA of Directive 2001/18*)

Answer 2: It should be advised to take nasal swabs at 5 and 10 days post intranasal vaccination. When a concurrent infection with a wild FHV occurs, then it should be examined whether recombination occurred. New rFHV(field)/FIVgag/env viruses should be analysed on their virulence and capacity to spread.

Answer 3: Yes, the proposed plan is adequate.

Answer 4: Not completely. In case of unexpected effect only, cats will be kept indoor and an emergency plan with 3 different levels has been prepared. It might therefore be difficult to unequivocally identify the cause of a single unexpected event as been related to the vaccine and not to an independent event.

Answer 5: Not entirely

5.2. Which complementary measures could be considered to improve the monitoring plan?

Answer 1: Introduce one (not clear to me, *see technical dossier: information as required by Annex IIIA of Directive 2001/18*)

Answer 2: See 5.1.

Answer 3: None

Answer 4: To ask the owners keeping their cats inside during 3 weeks after the trial.

Answer 5: See point 5.8

5.3 What are the biosafety measures taken to avoid and/or minimise the spread of the GMO beyond the site of release/treated animal?

Answer 1: Not clear to me, apparently none.

Answer 2: None. Vaccinated animals/catteries should be isolated during a period of 14 days after vaccination.

Answer 3: The GMOs (TK deleted strains) have been engineered to attenuate the virulence of the parental strain and to reduce its ability to spread within the infected host and between infected and non infected animals.

Answer 4: Disinfection of surroundings of the places where the cats will be vaccinated, according to standard practices. The GMO is sensitive to conventional chemical agents.

Answer 5:

To keep the animals in a close environment during three weeks following vaccination. To avoid any stressing conditions able to induce virus reactivation.

There is no measure able to prevent reactivation of the virus during the entire life of the cat. However, an experiment performed by the applicant allows to postulate that reactivation and re-excretion is not likely to occur. The proposed measures could be therefore sufficient.

5.4. Which type of waste could be generated?

Answer 1: Cat litter, excreted biological fluids and excreta (not clear to me if virus positive - *see technical dossier: information as required by Annex IIIA of Directive 2001/18*)

Answer 2: Vaccine vials, droppers.

Answer 3: Vials and syringes.

Answer 4: Empty vials and applicator for intranasal inoculation, single use (one dose per vial).

Answer 5: Disposed vaccine vials.

5.5. Is the waste treatment proposed by the applicant satisfactory? Which complementary measures could be considered?

Answer 1: None; avoid contact with cat litter and excreta

Answer 2: No information available. What do they mean with the description "following internal procedures of recombinants"?

Answer 3: The proposed treatment is perfect.

Answer 4: Yes. Vials & applicators are collected on site of vaccination and destroyed and/or sterilized on site of production according to internal procedure for recombinant products.

Answer 5: Yes

5.6. When the applicant proposes emergency plans, do those plans assure the control of the potential negative effects?

Answer 1: None proposed.

Answer 2: Yes.

Answer 3: Yes, they do.

Answer 4: Yes.

Answer 5: Yes

5.7. Which complementary measures could be considered to improve the emergency plans?

Answer 1: Not clear to me; it seems to be (most probably correctly) assumed that the organism is safe for the environment in general; the purpose of the study seems to be an open target species vaccine safety study.

Answer 2: none

Answer 3: Based on the experience acquired from the use of other TK deleted alphaherpesviruses, one should be convinced that the emergency plan proposed is by far appropriate to the problem.

Answer 4: Considering the negligible risk, those are difficult to anticipate.

Answer 5: Nihil.

5.8. Do the measures to control the risk, as proposed by the applicant, allow to reduce the potential negative effects? Mention, eventually, the level of decrease of the risk and the level of feasibility of the proposed measure. If not, which other measures could be applied and what are the expected effects?

Answer 1: There are no real measures to control a potential risk. It seems to be (most probably correctly) assumed that the organism is safe for the environment in general; the purpose of the study seems to be an open target species vaccine safety study.

Answer 2: The monitoring, waste and emergency plans proposed by the applicant are satisfactory.
However, the applicant did not mention in the clinical protocol whether treated vet clinic patients (households) must be maintained/isolated or not inside the owner's house during the monitoring period. If vaccinated cats may walk outside, there is a likelihood of contamination of neighbouring cats (shedding/excretion).
As a complementary measure to improve the monitoring plan, oropharyngeal swabs should be taken at regular intervals to assess the GMO excretion/shedding in treated cats (cattery animals in particular).

Answer 3: See 5.1. and 5.3.

Answer 4: Yes, they do

Answer 5: Once again, the risk is negligible. The target species are 8 to 13-week-old cats but "older cats" are also considered in the trial. The safety of the vaccine is not documented in the dossier for pregnant cats as direct recipient but only as sentinels. Therefore, as the wild type FHV can induce abortion in some circumstances, an additional level of biosecurity could be obtained by avoiding the selection of pregnant female cats in the sampling.

Answer 6:

Since the vaccine virus will be applied in cats living in an open environment, in cats which may be infected by FeHV-1 at the time of vaccination, in cats which may be in contact with FeHV-1 infected cats during 2 weeks following vaccination, the risk of recombination and latent persistence in field conditions should be properly assessed by the applicant.
The trial should include a procedure which allows these points to be investigated, e.g.: nasal swab sampling before and after vaccination to monitor FeHV-1 infection and the likelihood of rise of recombinant viruses; regular nasal swabbing after the study over a one year period to monitor potential re-excretion of vaccine virus and rise of recombinant viruses.

Nevertheless, the biosafety assessment of this vaccine must also take into account the existing knowledge on live-attenuated alphaherpesvirus vaccines, either deleted in a non essential gene or not. Such vaccines can be found against BoHV-1 or pseudorabies virus (SuHV-1) with a deletion in the glycoprotein E gene, for example. These widely used vaccines have not been associated yet with the rise of recombinant viruses and the rise of strains exhibiting an increased virulence. It is therefore tempting to extrapolate these field observations to this application. It means that the trial can be conducted provided the applicant makes a good assessment of the risk of recombination and latent persistence of the TK- FeHV-1 in field conditions and includes in the protocol procedures able to identify these risks with a reasonable probability.

6. Question related to your expertise:

Context: The Biosafety advisory council needs to know if on some items it needs to seek advice of other experts.

Questions:

6.1 Do you think you don't have the needed expertise to answer some of the items in the risk evaluation? If yes, which one?

Answer 1: Question 2.3.

Answer 2: No

Answer 3: No. The problematic addressed in this dossier has been addressed earlier for other *alphaherpesvirinae.* The experiments performed with these various alphaherpesviruses all led to similar conclusions which can be summarized as follow: deletion of the TK gene drastically reduces the ability of the virus to spread in vivo and reduces its virulence. Consequently, any herpesvirologist has the required expertise to analyse this dossier.

Answer 4: No.

Answer 5: See point 2.3.

7. General or additional Comments

Comment 1: The completed EU document (*see technical dossier: information as required by Annex IIIA of Directive 2001/18*) seems to me of rather mediocre quality (e.g. identification techniques only very generally mentioned, not described); maybe this is because this seems to be a classical

vaccine target species safety study and the safety of the GMO vaccine strains is largely assumed (probably correctly as demonstrated in the very adequate ERA.

Comment 2: The dates proposed for release of the GMOs are not the same in the French and the English version of the dossier.

Comment 3: One of the GMO produced implicates the expression of a glycoprotein (Env). In term of safety, it could be interesting to know if this glycoprotein is incorporated in the envelope of the progeny virions. Indeed, in the latter case the transgene could affect the tropism of the virion. This is particularly important here as the Env glycoprotein has been shown to mediate the binding of the FIV virus. However, the experiments presented in the dossier demonstrates that the GMOs are safe for the target species.

References

Berlinski P., Forester N., Russell P., Stoeva M., Martin S., 2003, Inability to reactivate latent virus from tissues of cats previously vaccinated with recombinant feline herpesvirus vector vaccines expressing the FIV gag or env genes. Pharmacia&Upjohn Animal Health Study Report SR-0849-7923-2003-0XX. In progress.

Berlinski P., Forester N., Gibson J.K., Martin S., Reddy N.D., 2003, Shed/spread study to support licensure of feline immunodeficiency-rhinotracheitis vaccine, live feline herpesvirus vector, product code 16B1.R0 (unlicensed). Recombinant feline herpesvirus master seed vectoring the feline immunodeficiency virus gag gene (FHV-FIV-GAG MSV (X), 34285-6). Pharmacia&Upjohn Animal Health Study Report SR-0849-7923-2003-005. 7 February.

Berlinski P., Forester N., Gibson J.K., Martin S., Reddy N.D., 2003, Shed/spread study to support licensure of feline immunodeficiency-rhinotracheitis vaccine, live feline herpesvirus vector, product code 16B1.R0 (unlicensed). Recombinant feline herpesvirus master seed vectoring the feline immunodeficiency virus envelope gene (FHV-FIV-GAG MSV (X), 34285-5). Pharmacia&Upjohn Animal Health Study Report SR-0849-7923-2003-004. 7 February.

Crandell R.A., Rehkemper J.A., Niemann W.H., Ganaway J.R. & Maurer F.D., 1961, Experimental feline virus rhinotracheitis. *J. Am. Vet. Med. Ass*. **138**, 191.

Dean *et al.,* 1999, Journal of Virology, 73 (4): 2596-2603.

Dispas M., Schynts F., Lemaire M., Letellier C., Vanopdenbosch E., Thiry E., Kerkhofs P., 2003 Isolation of a glycoprotein E-deleted bovine herpesvirus type 1 strain in the field. *Vet. Rec*., **153**, 209-212.

Donaldson A.I. & Ferris N.P., 1976, The survival of some air-borne animal viruses in relation to relative humidity. *Vet. Microbiol*., **1**, 413.

Field H.J. & Wildy P., 1978, The pathogenicity of thymidine kinase-deficient mutants of herpes simplex virus in mice. *J. Hyg. Camb*. **81**, 267.

Fugita K., Maeda K., Yokoyama N., Miyazawa T., Kai C., Mikami T., 1998, In vitro recombination of feline herpesvirus 1. *Arch. Virol*., **143**, 25.

Gaskell R.M. & Povey R.C. (1982) Transmission of feline viral rhinotracheitis. *Vet. Rec*., **111**, 359.

Gaskell R.M., Dennis P.E., Goddard L.E., Cocker F.M. & Wills J.M., 1985, Isolation of felid herpesvirus 1 from the trigeminal ganglia of latently infected cats. *J. Gen. Virol*, **66**, 391.

Gogev S., Schynts F., Meurens F., Bourgot I., Thiry E., 2003, Biosafety of herpesvirus vectors. *Curr. Gene Ther*., **3**, 597-611.

Hoover E.A. & Griesemer R.A., 1971, Experimental feline herpesvirus infection in the pregnant cat. *Am. J. Path*., **65**, 173.

Kit S., Qavi H., Gaines J.D., Billingsley P.& McConnel S., 1985, Thymidine kinase- negative bovine herpesvirus type 1 mutant is stable and highly attenuated in calves. *Arch. Virol*., **86**, 63.

Kramer et al., 1991, Experimental infection of two dogs with a canine isolate of feline herpesvirus type 1. Vet Pathol 28:338-340

Maeda et al., 1998, Properties and functions of feline herpesvirus type 1 glycoproteins. J Vet Med Sci 60 (8) : 881-888

Marchiolli C.C., Yancey R.J., Wardley R.C., Thomsen D.R. & Post L.E., 1987, A vaccine strain of pseudorabies virus with deletions in the thymidine kinase glycoprotein X genes. *Am. J. Vet. Res*., **11**, 1577.

Martin S., Reddy N.D., Kausche F.M., Berlinski P.J., Chester S.T., Wood M.D., 2003, Efficacy evaluation: Vaccination of kittens for pivotal efficacy and duration of immunity using Feline Immunodeficiency Virus-Rhinotracheitis Vaccine, Live Feline Herpes Virus Vector, Product Code 16B1, R0 (unlicensed) as a single intranasal administration. Pharmacia&Upjohn Animal Health Protocol PR-0849-7923-E-SM-02-005. Study in progress.

Mengeling, W. L., 1991, Virus reactivation in pigs latently infected with a thymidine kinase negative vaccine strain of pseudorabies virus. *Arch Virol* **120**, 57-70.

Meurens F., Keil G.M., Muylkens B., Gogev S., Schynts F., Negro S., Wiggers L., Thiry E., 2004, Interspecific recombination between two ruminant alphaherpesviruses, bovine herpesviruses 1 and 5. *J. Virol*., **78**, 9828-9836.

Meurens F., Schynts F., Keil G., Muylkens B., Vanderplasschen A., Gallego P., Thiry E., 2004, Superinfection prevents recombination of the alphaherpesvirus bovine herpesvirus 1. *J. Virol.*, **78**, 3872-3879.

Povey R.C & Johnson R.H., 1970, Observations on the epidemiology and control of viral respiratory disease in cats. *J. Small Anim. Pract*., **11**, 485.

Rota P.A., Maes R.K., Evermann J.F., 1986, Biochemical and antigenic characterization of feline herpesvirus-1-like isolates from dogs. *Arch. Virol.*, **89**, 57-68.

Scott F.W., 1980, Viricidal disinfectants and feline viruses. *Am. J. Vet. Res*., **41**, 410.

Schynts F., Meurens F., Detry B., Vanderplasschen A., Thiry E., 2003, Rise and survival of bovine herpesvirus 1 recombinants after primary infection and reactivation from latency. *J. Virol*., **77**, 12535-12542.

Shields R.P. & Gaskin J.M., 1977, Fatal generalised feline viral rhinotracheitis in a young adult cat. J. *Am. Vet. Med. Ass.*, **170**, 439.

Spradbrow P.B., Carlisle C. & Watt D.A., 1971, The association of a herpesvirus with generalised disease in a young kitten. *Vet. Rec*., **89**, 542.

Tegtmeyer and Enders, 1969, Feline herpesvirus infection in fused cultures of naturally resistant human cells. Journal of Virology. 3: 469-476

Thiry E., 2002, Virologie clinique du chien et du chat. Editions du Point vétérinaire, Paris, 203 pages.

Thiry E., Meurens F., Muylkens B., Mc Voy M., Gogev S., Thiry J., Vanderplasschen A., Epstein A., Keil G.M., Schynts F., 2004, Recombination in alphaherpesviruses. *Rev. Med. Virol.*, accepted for publication.

Turner R., Shebab Z., Osborne K., Hendley J.O., 1982, Shedding and survival of herpes simplex virus from "fever blisters". *Pediatrics*, **70**, 547.

Van Pelt C.S. & Crandell R.A., 1987, Pancreatitis associated with a feline herpesvirus infection. *Comp. Anim. Prac.*, **1**, 7.

Verschoor *et al.,* 1995, A single mutation within the V3 envelope neutralization domain of feline immunodeficiency virus determines its tropism for CRFK cells, Journal of Virology, 69 (8): 4752-4757

Whetstone, C. A., Miller, J. M., Seal, B. S., Bello, L. J. & Lawrence, W. C., 1992, Latency and reactivation of a thymidine kinase-negative bovine herpesvirus 1 deletion mutant. *Arch Virol* **122**, 207-214

Yokoyama N., Meada K., Kawaguchi Y., Ono M., Tohya Y., Mikami T. (1995) Construction of the recombinant feline herpesvirus type 1 deleted thymidine kinase gene. *J. Vet. Med. Sci*., **57**, 709.

Yokoyama N., Maeda K., Tohya Y., Kawaguchi Y, Shin Y.S., Ono M., Ishiguro S., Fujikawa Y., Mikami T., 1996, Pathogenicity and vaccine efficacy of a thymidine kinase-deficient mutant of feline herpesvirus type 1 in cats. *Arch. Virol*., **141**, 481.