Biosafety Advisory Council

26 March 2004

Secretariat

O./ref.: WIV-ISP/BAC/2004_SC_112[1]
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Title: Advice of the Belgian Biosafety Council on the notification B/BE/03/B3 of the company Transgène for deliberate release in the environment of genetically modified organisms other than higher plants for research and development

Context

The notification B/BE/03/B3 was submitted by Transgène to the Belgian Competent Authorities in January 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 February 2, 2004.

The planned activity is a clinical trial on cancer patients with a genetically modified adenovirus designed to treat cancer patients. The title of the study is: **"Phase I/II multicentre study of TG1024 (Adenovirus Interleukin 2) in patients with metastatic melanoma or other advanced solid tumor cancers."**. The Belgian patients will be treated at the University Hospital Erasme in Brussels, where Prof. Thierry Velu, Department of Medical Oncology, will be the principal investigator. The study is already running in Switzerland. No other European country in involved in this trial.

Scientific evaluation

The dossier has been evaluated by a group of experts of the Biosafety Council. They 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] revised version of document BAC_2004_SC_109 as approved on 25 March 2004

1. Human adenovirus type 5 is a virus of the low biorisk class 2. The transmission of human adenovirus infection and disease varies from sporadic to epidemic. Direct or indirect transmission occurs from throat, faeces, eye or urine, depending on the virus serotype. Wild type adenoviruses cause harmless respiratory infections in humans and animals but adenoviruses have been isolated from immunocompromised patients and have contributed to their morbidity and mortality.

2. The vector is made replication-defective by deletions in E1 and E3 regions of the wild type adenoviral genome: all the necessary genes for correct replication and propagation have been deleted. E3-deleted vectors should be recognized and eliminated more readily by the immune system, hereby reducing vector persistence.
In the deleted E1 region, a fragment of DNA coding for Interleukin 2 (IL2) has been inserted. The sequence was obtained from a DNA fragment extracted from human peripheral blood lymphocytes. Interleukine 2 should simulate the immune system of the patient to eliminate cancer cells.

3. Due to the production method used the risk of generating replication-competent adenoviruses (RCA) is unlikely. Besides, all batches are rigorously tested for the presence of RCA by Transgène.

4. At the present time, 25 patients have been treated with TG1024 in Switzerland. Analysis has shown the good safety of the product administered every three weeks.
Up to the present, the principal side effects associated with the intra-tumour administration of TG1024 reported by patients are: fatigue, erythema and pain at the injection site, fever, chills, nausea and vomiting, loss of appetite, headaches and dizziness. The majority of the transduced adenoviral vector genomes essentially remain episomal, a state that minimizes the risk of insertional mutagenesis. In addition, since adenoviral vectors do not integrate, dividing cells will gradually loose the adenoviral vector.
For the patient, the risk due to the toxicity of IL-2 administrated locally in the tumour should be considered acceptable within the goals of this trial, as long as the IL-2 remains concentrated in the tumour.

5. If present, the low number of virus particles that become excreted may infect a few cells of the persons that are in contact, but this will cause no problem. No new virus will be produced and the low doses of IL2 that could be produced will cause no harm.
However, it cannot be ruled out that the recombinant adenovirus can exchange its genetic material during co-infection of the same human cell by a wild-type adenovirus and thus reacquires a replication capacity generating RCA. The probability of occurrence of this event is extremely low and would involve only a limited number of viral particles which would be rapidly eliminated by the immune system, and consequently would have no effects on health of the persons in contact with the treated patient after a putative horizontal transmission. In addition, the respect of confinement, carrying out of protection, control and monitoring measures could reduce significantly the likelihood of post-release dissemination of the vector to other persons. Since the presence of recombinant adenovirus has been demonstrated in

body fluids, mainly within the 24 hours following administration, the treated patient should be hospitalised for 24 hours and visits should be restricted to health care workers who should avoid any direct contact with body fluids and secretions.

6. TG1024 cannot be found in the natural environment due to its non replicative character (E1 deletion) and its incapacity to propagate in the natural environment. In addition, the human adenoviruses, namely HAdV-5 from which Ad-IL2 vectors are derived, is not pathogenic to animals and does not form tumours even in permissive animal models.

7. In the current study, intra-tumoral administration confines the Ad-IL2, at least the large amount of the latter, to a limited area near the injection site, limiting the risk of horizontal transmission i.e. transmission to other humans.
If inadvertent horizontal transmission occurs with replication-deficient adenoviral vectors or even with RCA, the risks would be minimal.

8. Based on preclinical studies, the risk of inadvertent germline gene transfer in patients enrolled in the further clinical trials with TG1024 is expected to be very low if any.

9. The possibility of the GMM to reconvert to his wild type form is extremely remote and unlikely to occur.

10. Gene transfer to other micro-organisms cannot be completely excluded but the risk is very low. Consequently the risk for human health and the environment is very low.

11. The magnitude of the as above identified potential risks is very low.

12. The monitoring, waste and emergency plans proposed by the applicant addresses the risks concerning potential adverse effects.

13. It would be advisable to reduce the person contacts of the patient to a minimum (no visit, especially with naive children) during a period of two weeks after the first injection, in order to exclude the possibility of the spread of a recombinant virus, that theoretically may be formed, to another person. During this period, we expect an appropriate immunological response shortly after the first injection that will eliminate eventually formed recombinant virus.
For broken ampoules, contacts with the skin and wounds, it would be the best to propose disinfectants which have been proven to be effective against adenoviruses.

Conclusion

Based on the scientific assessment, the Biosafety Advisory Council concludes that the risk of using this GMM in this clinical trial is, for human health and the environment, very low. It could still be reduced if some extra precautions are taken. Therefore, the dossier receives a positive advice under the following conditions:

1. The notifier and its investigators applies the protocol, the biosafety, monitoring and, if necessary, emergency measures as described in the dossier.

2. After each injection, the patients stays at least 24 hours in an individual room at the hospital until the level of viral vector in his body fluids has begun to decrease.

3. When back home, the patient is instructed to apply strict hygiene measures (hand washing, no exchange of dishes,..) during all the time of his participation in the study and, at least after the first injection, to reduce contacts with other persons to a minimum (no extra visits during the first 2 weeks, especially with naive children).

4. The waste placed in closed containers treated according to regular hospital procedure for infectious material is not placed in the same environment that the ones from virology department in order to avoid any contact with other viruses. It will be decontaminated separately but according to the same hospital procedure for infectious waste.

5. A complete and accurate treatment plan in the unexpected event of exacerbated immune or inflammatory reactions as in the Gellsinger's case is provided by the sponsor to the investigators.

6. The list of disinfectants proven to be effective against adenoviruses is provided by the sponsor to the investigator. A disinfectant from this list is made available in the rooms where the study medication will be handled.

Prof. Dr. ir. Dirk REHEUL

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

Annex : Expertise report. (ref: BAC_2004_GT_111)

Biosafety Advisory Council

Secretariat

O./ref.: WIV-ISP/BAC/2004_GT_111[1]
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Report of the Group of Experts mandated by the Biosafety Advisory Council
(meeting with the President on 17 February 2004)

March 26th, 2004

Evaluation of the notification B/BE/03/B3 of the company Transgène 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/ECand the Royal Decision of 18 December 1998

Coordinator's Summary report

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

[1] Revised version of document BAC_2004_GT_108 as requested on 25/03/2004

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INTRODUCTION

Dossier B/BE/03/B3 concerns a notification of the company Transgène 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 2 February 2004.

The notification concerns a project of a clinical trial in humans entitled: Phase I/II multicentre study of TG1024 (Adenovirus Interleukin 2) in patients with metastatic melanoma or other advanced solid tumor cancers.

The coordinator assisted by the Secretariat of the Biosafety Council has asked a dozen of experts among the members of the list of experts currently used for 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 as well as Annex 3 A. All the answers received are compiled in the document appended to this report (ref: BAC_2004_GT_106).
The coordinator summarized the different expert's comments and has added his personal view in order to build up this summary report. The conclusions are made by the coordinator based on the experts' comments.

Rem: The complete reference of the articles cited in the text can be found at the end of document BAC_2004_GT_106 in annex of this document.

1. Questions related to the characteristics of the recipient organism

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

Human adenovirus type 5 is a virus of the low biorisk class 2. There are only two non-human species that allow replication of HAdV-5 (wild type), namely the cotton rat and the hamster. The transmission of human adenovirus infection and disease varies from sporadic to epidemic. Direct or indirect transmission occurs from throat, faeces, eye or urine, depending on the virus serotype. . Wild type adenoviruses cause harmless respiratory infections in

humans and animals. However, adenoviruseses have been isolated from immunocompromised patients and have contributed to their morbidity and mortality. Seroprevalence of adenovirus infections was estimated to be high. Antibodies to HAdV-1, HAdV-2, and HAdV-5 are most common and are present in 40% to 60% of children (Brandt et al., 1969). Adenovirus-5 (Ad5) E1A proteins have transformation activity in rodent cells, but this has not yet been shown in human cell lines (Endter C, Dobner T.). The E3 protein has been shown to play a role in regulating expression of MHC molecules on the surface of infected cells, and deletion of this protein increases the response of cytotoxic T cells as well as polymorphonuclear leukocytes in the inflammatory response (Fessler SP, et al.). Human Ad5 from which most adenoviral vectors are derived, does not form tumours even in permissive animal models.

2. Questions related to the characteristics of the GMM

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

Multiple copies are introduced per cell depending on the multiplicity of infection (MOI) used. The expression of the transgene IL-2 is driven by the CMV promoter which a strong constitutive viral promoter resulting in a high expression of IL-2, The deletion of the E1 region results in a virus that only replicates in complementary cell lines. The modified organism used in this study ADTG13383 is HAdV-5 belonging to the first generation of adenoviral vectors. The vector ADTG13383 is made replication-defective by deletions in E1 (E1A and E1B) and E3 regions of the wild type adenoviral genome. Consequently, the risk of adenoviral vector-induced oncogenesis (which is E1a-dependent and has only been documented in vitro) in humans is extremely unlikely. The vector is devoid of E3. Since E3 contributes to immune-evasion by downregulating MHC class I expression and anti-viral TNF activity, E3-deleted vectors should be recognized and eliminated more readily by the immune system hereby reducing vector persistence. Consequently, by deleting E3 a more potent inflammatory response may be evoked than if E3 is present. Deletion of E1 can be overcome in vitro with either high multiplicities of infection (MOI) or through the action of cellular trans-activators (e.g. NF-IL6) with E1-like activity that could override the absence of transcription by the recombinant adenoviral vector (Imperiale et al.,1984; Spergel et al, 1992). For instance in HeLa cells, the complementation of E1A is presumably due to the endogenous human papillomavirus (HPV) 18 E7 gene product. Alternatively, other viruses such as Epstein Barr virus (EBV) have been shown to complement E1-defective adenoviruses. This implies that underlying viral infections or activation of NF-IL6 via other mechanisms (e.g. during an acute inflammatory response) might inadvertently activate replication of E1-deleted adenoviral vectors. Once viral gene expression is turned on, expression of the foreign viral antigens encoded by the first-generation adenoviral vector backbone can trigger destructive cellular immune responses mediated by cytotoxic T lymphocytes (CTLs) that eliminate the transduced cells (Yang et al,1994; Tang et al, 1996). The specific CTL-mediated elimination of transduced target cells are believed to contribute to the short-term transgene expression that is frequently observed with first-generation adenoviral vectors

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

There have been numerous clinical trials involving recombinant adenoviral vectors demonstrating their safety in humans and the environment. The risk for genetic recombination with a wild type virus present in the transduced cell is very low and, if it should happen, the result will not extend beyond the classical viremia of Ad5. All the necessary genes for correct replication and propagation have been deleted.
Risks to the patient appear to be minimal concerning insertion mutagenesis or virus recombination. However, the risks due to toxicity of the vector are still not clear. In one study, a patient died during safety testing of an Ad5 E1 and E4 deleted vector (Raper SE, et al.) when 6x1011 particles/kg were infused into the hepatic artery (the 17 previous patients in this trial showed none of the same symptoms). A violent immune response and an exaggerated inflammatory response associated with massive IL-6 and Il-10 release and activation of the innate immune system contributed to multi-organ dysfunction and ultimately the death of Jessi Gellsinger following injection of $6x10^{11}$ vp/kg (4.3x1013 vp). This fatality underlines the recurrent problem that animal studies do not necessarily predict human responses. It appears therefore that there may be significant differences in adenoviral toxicity among species and perhaps also among individuals. It is therefore essential that the stepwise dose administration proposed in this study with complete status assessment before the next progressively increasing dose is administered, be strictly followed for each individual enrolled in this trial. The risk due to the toxicity of IL-2 administrated locally in the tumour appears to be considerably less in human trials (Daniels GA, Galanis E.), and should be considered acceptable within the goals of this trial, as long as the IL-2 remains concentrated in the tumour.

The vectors used in this study will be injected intra-tumorally instead of via hepatic artery catheterization as in the Gellsinger case and will be injected over a longer time interval and at a lower dose per injection (3x1011 tp/injection x 12 or 18 injections = 3.6 or 5.4x1012 tp) compared to the Gellsinger dose (4.3x1013 vp). These conditions may decrease the apparent risk associated with the use of this GMO compared to the conditions used in the Gellsinger-OTC trial. So far, the study drug has been reasonably well tolerated in the enrolled patients, except for a grade III transient thrombocytopenia, leukopenia and neutropenia in one of the patients (patient # 025). Moreover, expression of a potent cytokine (IL-2) by the adenoviral vector may exacerbate the anti-viral response and it cannot be excluded that this may consequently amplify the inflammatory/innate immune response. No preclinical data were provided on hepatotoxicity, innate immune responses, thrombocytopenia of the Ad-CMV-IL2 vector (+/- DTIC) in non-human primates at doses similar or higher than the doses to be used in the patients. Whether pre-existing immunity to Ad (e.g. following natural exposure to Ad or repeated Ad administration) exacerbates or minimizes the side-effects associated with early generation Ad vectors, is still being debated.

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?

By the use of the PerC6 production cell line the risk of generating replication-competent adenoviruses (RCA) is unlikely. Besides, all batches are rigorously tested for the presence of RCA by Transgene. Row material of biological origin, such as trypsin and fetal bovine serum (FBS) used in the first step of the production until the master virus seed, were tested and treated by the supplier. Additional tests were also performed on trypsin (mycoplasma and porcin viral contaminants) and FBS (USA origin) (mycoplasma, sterility, BVD, bovine polyoma virus, endotoxin) upon Transgene S.A. request. USDA (United States Department of Agriculture) considers the USA to be free of bovine spongiform encephalopathy (BSE), and hence the serum is extracted from animals BSE free. However, caution is advised if we take in account the confirmed case of BSE in Canada on May 20, 2003. In addition, for production of each clinical lot from master virus seed, animal protein free medium was used instead of FBS for PER.C6 cell grown in suspension and trypsin was not used any longer.

3. Questions related to the risks for the environment

3.1 Immediate and/or delayed adverse effects on the human health resulting from the direct and indirect interactions between the GMM and the treated patient.

The effects that are observed are not due to virus replication (there is no virus replication) but due to the expression of some viral antigens and IL2. They consist mainly of fatigue, injection site erythema, injection site pain, pyrexia, rigors, nausea, vomiting, anorexia, dizziness, headache and transient lymphopenia. Two phase I studies were conducted with TG1021 in dose escalating manner. The patients suffering from digestive unresectable adenocarcinoma and from lung cancer received escalating single dose levels of TG1021 from 10^7 to 10^9 pfu by intra tumoral (IT) injection. No significant adverse effects were observed and treatment was generally well tolerated in both of study. In the current study, a phase I trial was conducted (Switzerland) in patients with metastatic melanoma or another type of solid tumour with TG1024 in dose escalating manner. The purpose of the trial was to determine the safety of repeated intra-tumoral injections of TG1024 and to determine the Maximal Tolerated Dose (MTD). The maximal dose of TG1024 planned in the present trial is $3x10^{11}$ pfu, about the same as the maximal dose administered (10^9 pfu) in clinical trials with previous generation products having good safety. At the present time, 25 patients have been treated with TG1024. Analysis has shown the good safety of the product administered every three weeks up to the dose of $3x10^{11}$ pfu. The protocol of the trial under way was amended to extend it to phase I/II in order to increase the rhythm of TG1024 injections (every 2 weeks, then every week) and to assess safety and efficacy of combining with a standard chemotherapy (dacarbazine) in melanoma patients. Up to the present, the principal side effects associated with the intra-tumour administration of TG1024 reported by patients are:

fatigue, erythema and pain at the injection site, fever, chills, nausea and vomiting, loss of appetite, headaches and dizziness. The majority of the transduced adenoviral vector genomes essentially remain episomal, a state that minimizes the risk of insertional mutagenesis. Since genomic integration is relatively rare, the risk of insertional mutagenesis and oncogenic transformation is substantially lower than when integrating vectors such as onco-retroviral vectors are employed. In addition, since adenoviral vectors do not integrate, dividing cells will gradually loose the adenoviral vector. Unlike the subgroups A and B, human HAdV-5 (subgroup C) does not form tumours even in permissive animal models. The E1 region is capable of transforming rodent cells in culture and some human cell types, albeit less frequently. The transforming potential of the E1 proteins is due to its interaction with cellular proteins involved in transcription and cell cycle regulation (e.g. p53). Also, the E4 ORF6 protein can substitute for E1B and cooperate with E1A to transform cells (Nevels et al., 1999). Since most adenoviral vectors have at least a deletion in the E1 region, and are consequently replication-deficient, the risk of adenoviral vector-induced oncogenesis in humans is very unlikely.

3.2 Immediate and/or delayed adverse effects on the human health resulting from the potential direct or indirect interactions between the GMM and people coming in contact with the treated patient and/or with the GMM (workers, patient relatives, etc...).

If present, the low number of virus particles that become excreted may infect a few cells of the persons that are in contact but this will cause no problem. No new virus will be produced. However, it cannot be ruled out that the recombinant adenovirus can exchange its genetic material during co-infection of the same human cell by a wild-type adenovirus and thus reacquires a replication capacity generating RCA. The probability of occurrence of this event is extremely low and would involve only a limited number of viral particles which would be rapidly eliminated by the immune system, and consequently would have no effects on health of the persons in contact with the treated patient after a putative horizontal transmission. In addition, the respect of confinement, carrying out of protection, control and monitoring measures could reduce significantly the likelihood of post-release dissemination of the vector to other persons. Since the presence of recombinant adenovirus has been demonstrated in body fluids, mainly within the 24 hours following administration, it should be advised to all contacts (health care workers and patient relatives or visitors) to avoid direct contact with body fluids and secretions within the first 24 hours following administration. More stringent measures are not necessary since infectious recombinant virus will no longer be present or only at extreme low numbers, which carry no health risk.
In view of the study design (see protocol), we assume the patients will be hospitalised for at least 24 hours. During this time span contacts with body fluids and secretions should be followed by washing off and disinfections of skin contact (as described in Item 8 of this dossier).

3.3 Immediate and/or delayed adverse effects on the environment and animal health resulting from the potential direct or indirect interactions between the GMM and other species.

TG1024 cannot be found in the natural environment due to its non replicative character (E1 deletion) and its incapacity to propagate in the natural environment. In addition, the human adenoviruses, namely HAdV-5 from which Ad-IL2 vectors are derived, is not pathogenic to animals and does not form tumours even in permissive animal models (Shenk, 2001).

3.4. Is there a significant probability that the GMM will spread from the patient to other persons or to the environment?

In the current study, intra-tumoral administration confines the Ad-IL2, at least the large amount of the latter, to a limited area near the injection site, limiting the risk of horizontal transmission. Nevertheless, dissemination of Ad-IL2 vector beyond the intra-tumoral injection site in several clinical trials has been already reported. Data available after the phase I trial of the current study, show the presence of viral DNA in the blood of patients, measured with specific tests (PCR), 1 to 2 hours after injections but rarely and only at low levels 24 hours after TG1024 intra-tumoral injection. The TG1021 DNA was also detected in blood samples only at day 0 post intra-tumoral injection and up to day 4 in patient receiving high doses. The faeces and tonsils were also positive up to day 4 and up to day 14 respectively. In the event that the vector or RCA would spread from the patient to other persons (via shedding, aerosols or blood transmission) the vector, RCA and/or transduced cells will likely be eliminated by the host's immune system unless the new recipient is immuno-compromised. In addition, some individuals that have naturally been exposed to wild-type adenovirus developed neutralizing antibodies (already mentioned high seroprevalence of neutralizing antibodies to HAdV-5 in human population) which constitute an additional barrier that protects against potential horizontal transmission of adenoviral vectors or RCA. This strongly suggests that if inadvertent horizontal transmission was to occur with replication-deficient adenoviral vectors or even with RCA, the risks would be minimal.

3.5 Are there potential risks to offspring, including vertical transmission?

The risk of insertion of adenoviral vector genomes into female germ cells during the course of somatic gene therapy was stringently tested in mice by injecting a high adenoviral vector dose into the ovary and by incubating naked oocytes with high titer adenoviral vectors (Gordon, 2001). Transgene expression was observed in the thecal portion of the ovary, with no staining seen in the oocytes. These data provide strong evidence that adenoviral vectors cannot readily transduce oocytes and that the risk of female germ-line transduction with such vectors is very low. Based on these preclinical studies, the risk of inadvertent germline gene transfer in patients enrolled in the further clinical trials with TG1024 is expected to be very low. The risk of inadvertent germline gene transfer resulting from accidental or occupational exposure to

adenoviral vectors or even RCA, which would typically involve a much lower dose than what is required in clinical protocols, would be even lower.

3.6. Are there any selective advantages or disadvantages conferred to the GMM compared to the parental organism?

The Ad-CMV-IL-2 vector is not expected to have any selective advantages over the wt adenovirus, on the contrary, since it is replication-deficient and expresses a gene that stimulates the immune systems it is more likely to have a selective disadvantage compared to the parental organism.

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

The vector ADTG13383 used in the current study was generated and propagated in PER.C6 complementing cell line to avoid any occurrence of RCA. The presence of RCA in batches destined to be used for clinical trials was tested by inoculation of human cell line A549 and RCA were not detected in each clinical lot used in clinical study. This possibility is extremely remote and unlikely to occur in view of the replicative disadvantage of such forms.

3.8 What is the possibility of the GMM to be complemented in vivo by replicating competent viruses and what are the possible consequences for the human health and environment?

The producers of this recombinant product admit that the virus that has been injected at high titers in the tumour can come into the blood and may be spread to different organs, including lungs. This means that it is theoretically possible that recombinant virus gets into a cell that is infected with replicating competent viruses (RCA). However, this chance is very low. Exposure of patients enrolled in clinical trials to RCA or to naturally occurring wild-type adenovirus could mobilize E1-deleted adenoviral vectors in vivo, which would increase the risk of horizontal transmission of the vector. Also, transcomplementation of adenoviral vector in vivo could induce potent inflammatory reactions and contribute to toxicity. However, the extent of vector mobilization by wild-type virus or RCA may be relatively limited at least when one bases oneself on animal models permissive for adenoviral replication. Replication of wild type virus takes place during a short period of one to two weeks and especially during childhood. The chance that this coincides with the injection is then of course very low. Further, what is the effect of recombinations that may occur? Only handicapped viruses will come out that do not replicate (E1 region/suicide-recombinants) or that do not show immune evasion (E3 region). It can be expected that the latter will normally be eliminated effectively by the raised immunity.

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

At present, there is no evidence, to our knowledge, that adenoviral vectors could transfer a transgene to other micro-organisms. However, this cannot be completely excluded. There is a possibility of recombination with other wild-type adenoviruses, but then again this risk is very low due their probability of being in the same cell at the same time.

4. Questions related to the risk assessment of GMM

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

The risk of horizontal and vertical transmission and the risk to the environment related to the Ad-CMV-IL2 vector is likely very low. However, the risk to the patient remains to be defined given several uncertainties related to the non-linear threshold effect, inter-patient variation, inter-species differences, adverse effects of DTIC, IL-2 and Ad combined and the lack of data on the preclinical safety evaluation of Ad-CMV-IL2 +/- DTIC in non-human primates.

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

Human adenoviruses belong to Risk Group 2 (Directive 2000/54EC of 18 September 2000). Since the GMM (TG1024) is E1 deleted and thus replication incompetent, potential risks related to the GMM should be categorized in class 2.

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

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 ?

The monitoring plan proposed by the applicant addresses the risks concerning potential adverse effects, in particular by evaluating the risk of horizontal and vertical transmission using PCR-based techniques in blood, tonsils, urine faeces from the human patients infected with the GMO. The safety with respect to the patient, particularly with respect to the known risks of adenoviral gene transfer will be closely monitored, in particular thrombocytopenia, liver toxicity, inflammatory responses, IL-6, DIC in addition to standard toxicological analysis and clinical observation immediately prior to, during and for 4 hrs after injection,

which should also allow for the identification of non-anticipated acute adverse effects in the patient.

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

No additional complementary measures regarding the monitoring of the risk of horizontal and vertical transmission to be required.

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

The GMO is released only for clinical use according to standard Good Clinical Practice conditions. Patients are kept in individual rooms to improve containment. All materials used for preparation and administration will be placed in closed containers and then decontaminated according to the regular hospital procedures for infectious material or according to the disinfection protocol for therapeutic units by Transgene S.A. Decontaminated wastes will be then discarded according to the regular way of destruction for hospital wastes. Appropriate antiviral product will be regularly used as a viral disinfectant at the hospital.

5.4 If you have identified potential risks to offspring, which complementary measures could be considered to minimise the risk? Should birth control measures be recommended to patients?

Birth control for several months is reasonable (has been applied to earlier protocols with similar constructs).

5.5. Which type of waste could be generated?

Waste that could be generated in post-release treatment, precisely, after preparation and intra tumoral administration of the vector in hospital, is composed of opened ampoules, tubes for dilution, syringes, needles, gloves, gauze dressing. This waste is placed in closed containers treated according to regular hospital procedure for infectious material. Lab coats, goggles, patient gown, bedding, are decontaminated according to the regular hospital procedure for infectious.

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

The waste treatment proposed by the applicant is satisfactory.

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

For broken ampoules, contacts with the skin and wounds, it would be the best to propose disinfectants which have been proven to be effective against adenoviruses.

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

None

5.9. 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?

It would be advisable to reduce the person contacts of the patient to a minimum (no visit, especially with naive children) during a period of two weeks after the first injection, in order to exclude the possibility of the spread of a recombinant virus, that theoretically may be formed (see 4.8.), to another person. During this period, we expect an appropriate immunological response shortly after the first injection that will eliminate eventually formed recombinant virus. Additional biosafety measures (others than the test of the vector batches on A549 cells to detect eventual generation of RCA), could be also applied, such as, for instance, safety validation and assessment of potential replication-competent adenovirus contamination and vector complementation in vivo when performed in animal models that allow replication of HAdV-5, namely the cotton rat and the hamster.

CONCLUSION

The non modified organism, the Human adenovirus 5 is a common cause of sporadic or epidemic respiratory tract infections in humans, mainly among children. Most adults are immunized. The course of the disease is usually benign except in immunocompromised individuals. It is classed as a biorisk class 2 pathogen.
The modified vector is made replication-defective by deletions in E1 (E1A and E1B) and E3 regions of the wild type adenoviral genome and by adding the Il-2 gene with a CMV promoter. E3-deleted vectors should be more readily recognized and eliminated by the immune system hereby reducing vector persistence. Even if in theory one can suggest that replicative capacity could be restored in vivo, the risk of such event is very unlikely if not negligible.

The risk due to the toxicity of IL-2 administrated locally in the tumour appears to be considerably less than the one observed in systemic injections and should be considered acceptable within the goals of this trial, as long as the IL-2 remains concentrated in the

tumour. So far, the study drug has been reasonably well tolerated in the enrolled patients, except for a grade III transient thrombocytopenia, leukopenia and neutropenia in one of the patients (patient # 025). However, it is therefore essential that the stepwise dose administration proposed in this study with complete status assessment before the next progressively increasing dose is administered, be strictly followed for each individual enrolled in this trial.

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 are satisfactory for the experts.

Concerning immediate and/or delayed adverse effects on humans resulting from the direct and indirect interactions between the GMM and the treated patient, the effects observed are those of the transgene and so far, no serious or significant adverse reactions have been documented in the trial.

Data available after the phase I trial of the current study, show the presence of viral DNA in the blood of patients, measured with specific tests (PCR), 1 to 2 hours after injections but rarely and only at low levels 24 hours after TG1024 after intra-tumour injection. This administration route likely reduces the risk of transmission to humans in contact with the patient, but does not exclude it. In order to decrease this risk, the treated patient should be hospitalised for 24 hours and visits should be restricted to health care workers who should avoid any contact with body fluids and secretions. The already mentioned high seroprevalence of neutralizing antibodies to HAdV-5 in human population will constitute an additional barrier that protects them against potential horizontal transmission of adenoviral vectors or RCA. If however transmission occurs, only a few cells of the persons will be infected and this will normally cause no problem. No new virus will be produced and the low doses of IL2 that could be produced will cause nor harm. It would however be recommended to reduce the person contacts of the patient to a minimum (no extra-visit, especially with naive children) during the first two weeks after at least the first injection in order to minimise the possibility of the spread of a recombinant virus, that theoretically may be formed to another person.

There is no known indication of risk of transmission of the GMM to animals or other micro-organisms in the trial procedure. However, additional biosafety measures (others than the test of the vector batches on A549 cells), should be applied, such as safety validation and assessment of potential replication-competent adenovirus contamination and vector complementation in vivo performed in animal models that allow replication of HAdV-5.

In conclusion, the risk of using this GMM in this clinical trial is extremely low and the proposed measures to reduce it are well described by the proposer.

A complete and accurate treatment plan in the unexpected event of exacerbated immune or inflammatory reactions as in the Gellsinger's case should be provided by the sponsor to the

investigators. The list of disinfectants proven to be effective against adenoviruses should be provided also in case of hazards by handling the vials and available in the rooms where the study will take place.

Finally, the waste placed in closed containers treated according to regular hospital procedure for infectious material should not be placed in the same environment that the ones from virology department in order to avoid any contact with other viruses. They should be decontaminated separately but according to the same hospital procedure for infectious waste.

Dr. P. Hermans
Coordinator of the Group of experts

Annex: Compilation of all the answers of the experts in charge of evaluating the dossier B/BE/03/B3 (ref: BAC_2004_GT_106)

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March 15th, 2004

Secr etariaat
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Answers of Experts in charge of evaluating the dossier B/BE/03/B3

Domains of expertise: virology, recombinant adenoviral vectors, gene therapy in oncology,
Mandate for the Group of Experts: mandate of the Biosafety Advisory Council (BAC) of February 17th, 2004
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Experts: Jacques De Grève (VUB), Marinee Chuah (KUL), Sacha Gogev (ULg), Hans Nauwynck (UG), Bart Neyns (VUB), Thierry Vanden Driessche (KUL), Vigo Van Tendeloo (UIA), Karen Willard-Gallo (ULB)
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/03/B3 concerns a notification of the company Transgène 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 2 February 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 human health and the environment related to the non modified parental organism (including pathogenic effects to human and animals)?

Answer 1: Wild type adenoviruses cause harmless respiratory infections in humans and animals and the GMM haslost its pathogenic character though not all of its immunogenic features.

Answer 2: 1.1. Human adenovirus type 5 is a virus of the low biorisk class 2. It replicates in the respiratory tract which leads to subclinical or clinical infections. Nasal congestion, coughing and fever due to rhinitis, pharyngitis, tonsillitis and sometimes pneumonia are the main symptoms. Most infections occur during childhood. Most adults are infection immune. It forms a low hazard for the human health.

Answer 3: Adenovirus infections occur worldwide in humans as well as in a variety of animal species. The transmission of human adenovirus infection and disease varies from sporadic to epidemic. Direct or indirect transmission occurs from throat, faeces, eye or urine, depending on the virus serotype. Human adenovirus infections are mostly asymptomatic, but can be associated with diseases of the respiratory, ocular, and gastrointestinal systems. Less frequently, adenoviruses can infect the urinary bladder or liver. They may also occasionally cause diseases in other organs, such as the pancreas, myocardium or central nervous system (Horwitz, 2001). Adenoviruses have been isolated from immunocompromised patients and have contributed to their morbidity and mortality. The pattern of diseases caused by adenoviruses often correlates well with the viral serotype and the age (children or adults) of the susceptible population. Adenovirus infections typically do not cause permanent problems or death. The exceptions are infection in immunodeficient patient and acute respiratory disease (ARD), which can be fatal. Among the human adenoviruses belonging to subgenus C, the members HAdV-1 (human adenovirus type 1), HAdV-2 and HAdV-5 are regarded as endemic. They are responsible for respiratory infections in children. HAdV-5 is also involved in pertussis-like syndrome, intussusception and in hepatitis in pediatric transplant recipients (Sturdy, 1971; Potter, 1964; Cames *et al.,* 1992). Seroprevalence of adenovirus infections was estimated to be high. Antibodies to HAdV-1, HAdV-2, and HAdV-5 are most common and are present in 40% to 60% of children (Brandt *et al.,* 1969). These serotypes are capable of establishing persistent asymptomatic infections in tonsillar and adenoid tissues (Neumann *et al.,* 1987; Macek *et al.,* 1994). Shedding of the virus can occur for months or years after the initial infection. The high occurrence and persistence of adenovirus genome sequences in human lymphoid cells was demonstrated (Andiman *et al.,* 1982; Abken *et al.,* 1987; Horvath *et al.,* 1986; Flomenberg *et al.,* 1996) and an indication that a region other than E1A is involved in persistence, was also reported (Araujo *et al.,* 2001).

There are no effective medications to treat adenovirus infection. However, it was reported recently that an antisense oligodeoxynucleotides (ODNs) targeted to the E1A gene can specifically inhibit HAdV-5 infection *in vitro*, suggesting a potential therapeutic role for antisense ODNs in adenovirus infection (Whitehead *et al.,* 2003).

Generally, with few exceptions, the human adenoviruses are not pathogenic to animals, and animal adenoviruses are only pathogenic within the species of origin (Horwitz, 2001). There are only two non-human species that allow replication of HAdV-5 (wild type), namely the cotton rat and the hamster. Non modified parental HAdV-5 causes pulmonary histopathology in these permissive animal models equivalent to the corresponding human disease (Pacini *et al.*, 1984; Hjorth *et al.*, 1988); therefore, biosafety validation and assessment of potential replication-competent adenovirus contamination *in vivo* can be performed in these species (Oualikene *et al.,* 1995).

The E1 region of most if not all adenoviruses is capable of transforming rodent cells in culture and some human cell types, albeit less frequently. Unlike replication-competent adenoviruses belonging to the subgroups A and B (e.g. HAdV-12, HAdV-18, HAdV-31) which are highly oncogenic, producing tumors in rodents, HAdV-5 from which most adenoviral vectors are derived, does not form tumors even in permissive animal models (Shenk, 2001). The transforming potential of the E1 proteins is due to its interaction with cellular proteins involved in transcription and cell cycle regulation (e.g. p53). In addition, the E4 ORF6 protein may have some oncogenic properties (Moore *et*

al., 1996; Nevels *et al.,* 1999). Since most adenoviral vectors have at least a deletion in the E1 region, and are consequently replication-deficient, the risk of adenoviral vector-induced oncogenesis in humans is very unlikely. Furthermore, despite their transforming potential *in vitro*, there is no clinical evidence, to my knowledge, that wild-type adenoviruses are causally related to oncogenesis in humans. In addition, it was suggested that the lack of telomerase expression in somatic cells is, at least in part, responsible for the block to adenovirus oncogenicity in humans (Hahn et al., 1999).

Answer 4: Adenovirus infections with the 51 different serotypes recognized to date have few pathogenic signs and symptoms, and thus require a variety of laboratory-based procedures to confirm infection. These viruses have the ability to target various organs with relative serotype specificity and can cause diverse manifestations including serious life-threatening diseases in the immunocompromised host characteristic of the organ(s) involved. Adenovirus-5 (Ad5) E1A proteins have transformation activity in rodent cells, but this has not yet been shown in human cell lines (Endter C, Dobner T.). The E3 protein has been shown to play a role in regulating expression of MHC molecules on the surface of infected cells, and deletion of this protein increases the response of cytotoxic T cells as well as polymorphonuclear leukocytes in the inflammatory response (Fessler SP, et al.).

Answer 5: The hazards are limited to transient viral infection/illness of low severity in humans without a severely immunocompromised status.

Answer 6: Limited and well-known hazard of non-modified parent. Well described in the documents

Answer 7: The GMO is derived from a group C adenovirus (Ad) serotype 5. The risks associated with the parental wild-type Ad are well known and have been adequately described in this gene therapy protocol. Ad5 causes a relatively benign upper respiratory tract infection with no significant mortality. Ad5 and other Ad serotypes have been used to vaccinate volunteers without any significant side effects. Ad5 is not oncogenic in human and animals and is capable of replicating in human cells and cells of some other animals, although mouse cells are refractory to Ad5 replication. Hence the wt Ad5 is relatively benign, at least when exposed to man via the natural route of exposure and at the typically low doses. This issue has been satisfactorily addressed in this application.

Nevertheless, the E1 region of most, if not all adenoviruses is capable of transforming rodent cells in culture and some human cell types, albeit less frequently. Only replication-competent adenoviruses belonging to the so-called subgroup A and B (e.g. Ad12, Ad7) are capable of inducing tumors in animals, particularly in hamsters. In contrast, human Ad5 from which most adenoviral vectors are derived, does not form tumors even in permissive animal models, which seems to be at least partly due to the elimination of the virus and/or virally-infected cells by the host's immune system The transforming potential of the E1 proteins is due to its interaction with cellular proteins involved in transcription and cell cycle regulation (e.g. p53). In addition, the E4 ORF6 protein may have some oncogenic properties. Despite their transforming potential in vitro, there is no clinical evidence, to our knowledge, that wild-type adenoviruses are causally related to oncogenesis in humans.

2. Questions related to the characteristics of the GMM:

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: Adenoviral transduction results in an episomal transgene expression for a prolonged but not permanent period since there is no evidence for host genome integration of the viral construct (Volpers et al, J Gene Medicine 2004;6 Suppl 1:S164-71). Multiple copies are introduced per cell depending on the multiplicity of infection (MOI) used. The expression of the transgene IL-2 is driven by the CMV promoter which a strong constitutive viral promoter resulting in a high expression of IL-2, although some groups have experienced a downshutting of the CMV promoter activity after several weeks in vivo (Gaetano et al, Gene Ther. 2000 Oct;7(19):1624-30).

Answer 2: The deletion of the E1 region results in a virus that only replicates in complementary cell lines. In non-complementary cells such as susceptible cells of the respiratory tract, the virus infects cells but does not produce new virus and thus does not spread any more. This makes the product very safe.

Answer 3: The modified organism used in this study ADTG13383 is HAdV-5 belonging to the first generation of adenoviral vectors. The vector ADTG13383 is made replication-defective by deletions in E1 (E1A and E1B) and E3 regions of the wild type adenoviral genome. Unlike the wild type HAdV-5, the ADTG13383 has lost its replication capacity, cannot propagate in human and animal cell because of the failure in the expression of E1 gene, and thus its low pathogenicity. It grows only in cell lines (PER.6 in this study) that express the proteins encoded by E1 region. The E1A proteins are the first to be expressed *de novo* upon infection with wild-type adenovirus and constitute an essential master-switch to turn on adenoviral gene expression. Expression of E1A initiates adenoviral replication and activates adenoviral transcription. In addition, E1A stimulates the host cell to enter S phase. The E1B proteins stimulate viral mRNA transport while blocking host mRNA transport. E1B blocks E1A-induced apoptosis (White *et al.*, 1991). The E3 region encodes many different types of proteins that are involved in invasion of the host immune system (Wold and Gooding, 1989). The E3 gp19 kD protein binds to MHC class I molecules, causing their retention in the endoplasmic reticulum. Cells with reduced or no MHC-I expression should then become relatively resistant to MHC-class I dependent CTLs. Other proteins encoded by the E3 region inhibit cytolysis and inflammation induced by TNF (Krajcsi *et al.*, 1996; Sparer *et al.*, 1996).

Answer 4: An important obstacle to the use of adenovirus vectors has been their immunogenicity, and thus disabled gene-deleted adenovirus vectors have been engineered to reduce toxicity and circumvent cytotoxic T-cell responses against transduced cells. In the Ad-IL2 construct, removal of the Ad5 E1 and E3 proteins renders the virus non-replicative and therefore a producer cell line is required for virus production. In the place of the deleted E1 gene (the transforming region), they have added a CMV promoter, an intron from the human β globin gene, a cDNA for the human IL-2 gene, a polyA site from SV-40, and a fragment of the Ad5 polypeptide IX, none of which should render the vector replication competent or restore its oncogenic potential.

Answer 5: In short: abs ence of the possibility for replication in the absence of complementation. Expression of the Il-2 transgene by transduced cells. The information provided by the sponsor regarding this topic is complete and adequate.

Answer 6: Result is a replication incompetent organism containing an axogeneous gene, rhIL-2. Upon infection in celles capable of locally producing rhIL-2, a substance which can also be given systemically and has been registered as a legitimate drug for the treatment of renal cell cancer.

Answer 7: Compared to wt Ad, the first-generation DE1DE3-deleted Ad vector used in this study is significantly less pathogenic since it is replication deficient due to deletion of the E1a gene. Consequently, the risk of adenoviral vector-induced oncogenesis (which is E1a-dependent and has only been documented in vitro) in humans is extremely unlikely. The vector retains the E1b region which allows the infected cells to resist apoptosis and TNF. The vector is devoid of E3. Since E3 contributes to immune-evasion by downregulating MHC class I expression and anti-viral TNF activity, E3-deleted vectors should be recognized and eliminated more readily by the immune system hereby reducing vector persistence. Consequently, by deleting E3 a more potent inflammatory response may be evoked than if E3 is present.

Despite deleting the E1 "master-switch" gene, this DE1DE3-deleted Ad vector still retains and expresses early and late viral genes that can contribute to vector toxicity and immune responses. Although it was initially assumed that deletions of E1 sequences in first-generation adenoviral vectors would suffice to abrogate expression of other early and late viral genes, this has proven not to be the case. Deletion of E1 can be overcome in vitro with either high multiplicities of infection (moi) or through the action of cellular trans-activators (e.g. NF-IL6) with E1-like activity that could override the absence of transcription by the recombinant adenoviral vector [Imperiale et al.,1984; Spergel et al, 1992]. For instance in HeLa cells, the complementation of E1A is presumably due to the endogenous human papillomavirus (HPV) 18 E7 gene product. Alternatively, other viruses such as Epstein Barr virus (EBV) have been shown to complement E1-defective adenoviruses. This implies that underlying viral infections or activation of NF-IL6 via other mechanisms (e.g. during an acute inflammatory response) might inadvertently activate replication of E1-deleted adenoviral vectors. Once viral gene expression is turned on, expression of the foreign viral antigens encoded by the first-generation adenoviral vector backbone can trigger destructive cellular immune responses mediated by cytotoxic T lymphocytes (CTLs) that eliminate the transduced cells [Yang et al,1994; Tang et al, 1996]. The specific CTL-mediated elimination of transduced target cells are believed to contribute to the short-term transgene expression that is frequently observed with first-generation adenoviral vectors. However, the immune response against the transgene product in itself can limit the duration of expression [Tripathy et al, 1996; Christ et al, 1997], at least in some tissues. In some cases, expression of a foreign protein by means of a first-generation adenoviral vector could even break immune tolerance and trigger an auto-immune response against homologous self proteins. In the absence of specific cellular or humoral immune responses, expression of at least some transgenes using first-generation adenoviral vectors is transient indicating that other CTL-independent mechanisms contribute to the decline in transgene expression. These mechanisms may be related to activation of the innate immune system and/or direct toxicity of the vector particles [Schnieder et al, 1998] that may be associated, at least partly, with IL-6 release. Typically, transduction with early-generation DE1DE3-deleted Ad vectors result in transient transgene expression due primarily to the adaptive and innate host immune response. The vector used in this study also contains the IL-2 gene.

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

Answer 1: Yes, there has been numerous clinical trials involving recombinant adenoviral vectors demonstrating their safety in humans and the environment. As there is no integration of the genetic material, there is no risk for insertional mutagenesis. The risk for genetic recombination with a wild type virus present in the transduced cell is very low and, if it should happen, the result will not extend beyond the classical viremia of Ad5. All the necessary genes for correct replication and propagation have been deleted.

Answer 2: Yes

Answer 3: The genome of HAdV-5 is entirely sequenced and genomic organisation of HAdV-5 based vector is well known. In this study, the generation of ADTG13383 is detailed and the obtained vector is well characterised using restriction and sequence analyses. The resulting genomic structure is in compliance with predicted pattern based on vector construction. The expression and the functionality of IL2 encoded by the vector vehicled transgene in 293 cell line, were also measured.

In summary, the genetic modifications in obtained vector ADTG13383 are sufficiently characterised for the purpose of evaluating the risks to the human health and the environment.

Answer 4: Risks to the patient appear to be minimal concerning insertion mutagenesis or virus recombination. However, the risks due to toxicity of the vector are still not clear. In one study, a patient died during safety testing of an Ad5 E1 and E4 deleted vector (Raper SE, et al.) when $6x10^{11}$ particles/kg were infused into the hepatic artery (the 17 previous patients in this trial showed none of the same symptoms). This fatality underlines the recurrent problem that animal studies do not necessarily predict human responses, and that there can be significant variation between individual responses to the toxicity of replication defective adenovirus vectors. It is therefore essential that the stepwise dose administration proposed in this study with complete status assessment before the next progressively increasing dose is administered, be strictly followed for each individual enrolled in this trial.

The administration of high doses of IL-2 has been employed in a number of human cancer therapy trials and these studies clearly show that the toxicity, particularly with systemic administration, can be significant (Bleumer I, et al.; Buzaid AC, Atkins M.). Animal studies using Ad-IL2 suggest that there is also IL-2 toxicity with systemic virus administration, particularly in cats. The risk due to the toxicity of IL-2 administrated locally in the tumor is appears to considerably less in human trials (Daniels GA, Galanis E.), and should be considered acceptable within the goals of this trial, as long as the IL-2 remains concentrated in the tumor. Patient serum IL-2 levels need to be monitored to insure that unacceptable toxic levels are not unexpectedly attained in a given individual.

Answer 5: Yes

Answer 6: Affirmative

Answer 7: The risks are most significant with respect to the patients themselves, whereas the risk for the environment (horizontal and vertical transmission) is limited. Although the vector used in this

study is devoid of E1, preclinical studies in rodent models have indicated that this type of early-generation Ad vector results in higher acute and chronic toxicities compared to "gutted" adenoviral vectors that are devoid of residual adenoviral genes. Moreover, it is well established that mice are relatively refractory to the toxic effects of early-generation adenoviral vectors compared to non-human primates and possibly in human, when comparable vector doses/kg are employed. It appears therefore that there may be significant differences in adenoviral toxicity among species and perhaps also among individuals. This makes it particularly difficult to extrapolate safety data from mice to larger animal models or patients. One of the challenges of using early-generation adenoviral vectors in human gene therapy applications is the relatively narrow therapeutic window and the non-linear dose-response (so-called "threshold" effect) which is at least partly due to the innate immune response. A violent immune response and an exaggerated inflammatory response associated with massive IL-6 release and activation of the innate immune system contributed to multi-organ dysfunction and ultimately the death of Jessi Gellsinger following injection of 6x1011 vp/kg (4.3x1013 vp).

The vectors used in this study will be injected intra-tumorally instead of via hepatic artery catheterization as in the Gellsinger case and will be injected over a longer time interval and at a lower dose per injection (3x1011 tp/injection x 12 or 18 injections = 3.6 or 5.4x1012 tp) compared to the Gellsinger dose (4.3x1013 vp). These conditions may decrease the apparent risk associated with the use of this GMO compared to the conditions used in the Gellsinger-OTC trial So far, the study drug has been reasonably well tolerated in the enrolled patients, except for a grade III transient thrombocytopenia, leukopenia and neutropenia in one of the patients (patient # 025).

Nevertheless, there are still some unknowns that may increase the apparent risk associated with the use of this GMO. In particular, it is important to exclude that at the intensified dose regimen in the current trial design, any adenoviral vector "spill-over " from the tumor into the circulation does not cause significant hepatoxicity, thrombocytopenia and/or exacerbated innate immune system activation, reminiscent of the Gellsinger incident. This risk cannot be adequately addressed in mouse models (see above). In view of the non-linear dose response, inter-individual patient variation (cfr. Wilson OTC trial), species differences (mouse versus non-human primates and human), and the significant toxicity, morbidity and mortality in the cat model, there is a significant risk associated with the use of this Ad-CMV-IL2 vector in patients. Moreover, the anti-cancer drug dacarbazine and IL-2 by themselves are known to trigger similar type of side-effects (e.g., thrombocytopenia, hepatotoxicity). Grade III thrombocytopenia related to the study drug was apparent in patient 025 who received the maximum dose. Moreover, expression of a potent cytokine (IL-2) by the adenoviral vector may exacerbate the anti-viral response and it cannot be excluded that this may consequently amplify the inflammatory/innate immune response following exposure to early generation Ad vectors. Unfortunately, no preclinical data were provided on hepatotoxicity, innate immune responses, thrombocytopenia of the Ad-CMV-IL2 vector (+/- DTIC) in non-human primates at doses similar or higher than the doses to be used in the patients. Another unknown is whether pre-existing immunity to Ad (e.g. following natural exposure to Ad or repeated Ad administration) exacerbates or minimizes the side-effects associated with early generation Ad vectors, which is still being debated.

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: By the use of the PerC6 production cell line the risk of generating replication-competent adenoviruses (RCA) is unlikely to non existing because there are no homologous regions shared by the viral construct present in the cell line and the recombinant AdV genome. Besides, all batches are rigorously tested for the presence of RCA by Transgene.

Answer 2: Yes

Answer 3: Row material of biological origin, such as trypsin and fetal bovine serum (FBS) used in the first step of the production until the master virus seed, were tested and treated by the supplier. Additional tests were also performed on trypsin (mycoplasma and porcin viral contaminants) and FBS (USA origin)(mycoplasma, sterility, BVD, bovine polyoma virus, endotoxin) upon Transgene S.A. request. USDA (United States Department of Agriculture) considers the USA to be free of bovine spongiform encephalopathy (BSE), and hence the serum is extracted from animals BSE free. However, caution is advised if we take in account the confirmed case of BSE in Canada On May 20, 2003. In addition, for production of each clinical lot from master virus seed, animal protein free medium was used instead of FBS for PER.C6 cell grown in suspension and trypsin was not used any longer.

Numerous quality control tests were performed by applicant on PER.C6 complementation cell line, pre-master virus seeds (1-3), master virus seed and final product, but no evidence of bacteria, viruses, fungi, mycoplasma or PrP^{Sc} (only for PER.C6 cells) was reported. Control tests performed on A549 cells to detect the presence of RCA in the final product TG1024 were also negative.

At present, I consider that all measures needed to detect and eliminate any contaminating viruses 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, were taken.

Answer 4: Yes

Answer 5: Yes.

Answer 6: Affirmative

Answer 7: Adequate steps have been taken to reduce the risk of generating replication-competent adenoviruses (RCA) by virtue of the use of the PER.C6 complementation cell line in which there is no homologous overlap between vector and helper. In addition, there is reduced use of proteins from animal origin. The necessary steps are taken to detect possible RCA and other micro-organisms.

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 GMM and other organisms.

Questions

3.1 Immediate and/or delayed adverse effects on the human health resulting from the direct and indirect interactions between the GMM and the treated patient.

Answer 1: Aside from the the normal immunogenic response to the remaining adenoviral genes in the GMM that could impede re-administration of the same GMM due to clearing by a memory immune response against the GMM, the expression of high local concentrations of IL-2 could lead to flu-like symptoms which cannot be considered as adverse events.

Answer 2: The effects that are observed are not due to virus replication (there is no virus replication) but due to the expression of some viral antigens and IL2. They consist mainly of fatigue, injection site erythema, injection site pain, pyrexia, rigors, nausea, vomiting, anorexia, dizziness, headache and transient lymphopenia. Systemic administration of IL2 gives low blood pressure, renal and hepatic toxicity, respiratory difficulties, fever, nausea, vomiting, loss of weight, erythema and skin rash and anemia. These effects are reversible when treatment is stopped.

Answer 3: Adenovirus vector not only has an advantage of high efficiency of gene transduction, but also has disadvantage of direct toxicity and immunogenicity both *in vitro* and *in vivo* when used at high multiplicities of infection (Morral *et al.,* 1997). Many clinical trials are based on first and second generation adenoviral vectors for a variety of genetic, acquired and complex diseases. Recently, a clinical trial based on gutless adenoviral vectors has been initiated. Administration by aerosol of adenoviral vectors in a patient suffering from cystic fibrosis led to acute respiratory distress syndrome (ARDS). This was most likely due to the inflammatory response that was evoked by this early generation adenoviral vector and the trial was discontinued. Other trials revealed side effects related to stage III transitory hepatic impairment, hemostatic problems related to thrombocytopenia, hypotension and tachycardia. The most severe reaction was observed when an 18-year old patient suffering from partial ornithine transcarbamylase (OTC) deficiency, was injected with an E1/E3/E4-deleted adenoviral vector containing the OTC gene (Raper *et al.,* 2003). A vector dose of $3.8x10^{13}$ vector particles was injected into the patient by hepatic artery catheterization. Post-injection, the patient rapidly developed fever, nausea, vomiting, muscular pain and tachycardia. Disseminated intravascular coagulation and hepatic impairment occurred followed by ARDS, coma and death 4 days post-injection. Post-mortem examination revealed infiltration and inflammation of the lungs that likely caused the ARDS, as well as anoxia of brain, liver, spleen and kidneys. The vector was present in all organs, including testis, suggesting that intravascular injection is undesirable. Abnormally high serum IL-6 levels were detected in the patient following administration of the vectors.

It was reported that the capsid binding and uptake induce the initial inflammatory events (Liu et al. 2003), so all generation of adenovirus based vector, even the gutless, induce this phase of inflammation that could lead to serious and fatal adverse events during the clinical trial. Nevertheless, it was reported recently that an adenovirus deleted for the E1A 289R protein (the coding region for E1A was replaced with cDNA encoding only the E1A 243R protein) was noninflammatory, and inhibited edema induced by empty virus particles in mice (Schaack *et al.,* 2004). These findings hold promise for the development of optimal replication defective and helper dependent gene therapy

vector. The use of this adenovirus as helper could inhibit the inflammation induced by both the contaminating helper virus and the helper dependant vector.

Toxicity studies were carried out with the previous two generation of Ad-IL2 (adenovirus expressing human Interleukin 2) vectors, namely TG1021 (E1 and E3 deleted containing the human IL2 under the control of the MLP promoter) and TG1022 (E1, E3 and E4 deleted containing the human IL2 under the control of the RSV promoter). Two phase I studies were conducted with TG1021 in dose escalating manner. The patients suffering from digestive unresectable adenocarcinoma and from lung cancer received escalating single dose levels of TG1021 from 10^7 to 10^9 pfu by intra tumoral (IT) injection. No significant adverse effects were observed and treatment was generally well tolerated in both of study. One phase I study was also conducted with TG1022 in patient with colorectal cancer. A single administration of 10^7 pfu into hepatic metastases was well tolerated. Nevertheless, site redness, swelling and discomfort, fatigue as well as asthenia and inferior limb pain were reported with these vectors.

In the current study, a phase I trial was conducted (Switzerland) in patients with metastatic melanoma or another type of solid tumour with TG1024 in dose escalating manner. The purpose of the trial was to determine the safety of repeated IT injections of TG1024 and to determine the Maximal Tolerated Dose (MTD). The maximal dose of TG1024 planned in the present trial is $3x10^{11}$ pfu, about the same as the maximal dose administered (10^9 pfu) in clinical trials with previous generation products having good safety. At the present time, 25 patients have been treated with TG1024. Analysis has shown the good safety of the product administered every three weeks up to the dose of 3x10 11 pfu. The protocol of the trial under way was amended to extend it to phase I/II in order to increase the rhythm of TG1024 injections (every 2 weeks, then every week) and to assess safety and efficacy of combining with a standard chemotherapy (dacarbazine) in melanoma patients. Up to the present, the principal side effects associated with the intra-tumour administration of TG1024 reported by patients are: fatigue, erythema and pain at the injection site, fever, chills, nausea and vomiting, loss of appetite, headaches and dizziness.

The properties of the transgene present in the adenoviral vectors determine also the biosafety profile of the vectors. Some proteins such as coagulation factors are therapeutic over a wide concentration range and consequently the expression levels do not need to be tightly regulated. The risk potentiation by the corresponding transgene in those cases is minimal, not only in the patients undergoing gene therapy, but also following horizontal transmission of such a vector. However, for other genes encoding hormones, growth factors, growth factor receptors, or potentially toxic or oncogenic proteins, over expression may be undesirable. The systemic (intravenous or subcutaneous) administration of IL2, causes severe toxic effects, making it a good candidate for gene therapy. The intra-tumour administration of the gene expressing IL2 via an adenovirus limits the toxic effects of the protein in the bloodstream (Stewart *et al.,* 1999), at the same time as maintaining a locally high expression inside the tumour, enabling it to exert its anti tumour effect. No major adverse effect have been observed in patients treated with Ad-IL2 (Stewart *et al.,* 1999) (TG1021, TG1022 in the previous studies and TG1024 in the ongoing study), reported to be related to IL2.

The majority of the transduced adenoviral vector genomes essentially remain episomal, a state that minimizes the risk of insertional mutagenesis. Since genomic integration is relatively rare, the risk of insertional mutagenesis and oncogenic transformation is substantially lower than when integrating vectors such as onco-retroviral vectors are employed. In addition, since adenoviral vectors do not integrate, dividing cells will gradually loose the adenoviral vector. Unlike the subgroups A and B, human HAdV-5 (subgroup C) does not form tumors even in permissive animal models. The E1 region is capable of transforming rodent cells in culture and some human cell types, albeit less frequently. The transforming potential of the E1 proteins is due to its interaction with cellular proteins involved in transcription and cell cycle regulation (e.g. p53). Also, the E4 ORF6 protein can substitute for E1B

and cooperate with E1A to transform cells (Nevels *et al.*, 1999). Since most adenoviral vectors have at least a deletion in the E1 region, and are consequently replication-deficient, the risk of adenoviral vector-induced oncogenesis in humans is very unlikely. Furthermore, despite their transforming potential *in vitro*, there is no clinical evidence, to my knowledge, that wild-type HAdV-5 is causally related to oncogenesis in humans. In addition, the experiment conducted by Hahn et al. (1999) suggested that the lack of telomerase expression in somatic cells is, at least in part, responsible for the block to adenovirus oncogenicity in humans.

Answer 4: Potential toxicity due to the administration of the modified Ad5 virus itself and/or its production of IL-2 (as discussed above).

Answer 5: Immediate adverse effect both from a reaction of the patient against the immunogenic vector and/or the expressed transgene can not be excluded (see also the information provided by the sponsor on the incidental acute and severe inflammatory reactions that have been reported in patients injected by recombinant adenoviral vectors). The immediate adverse effects resulting from the reaction of the patient against the recombinant adenoviral vector should be estimated as acceptable if (as intended in this study) injection of the vector will be done in tumoral masses. Such administration has not been reported to result in acute life-threatening events in previous studies. Immediate adverse effects from expression of the transgene can be expected since it is known that Il-2 administered as a cytokine (outside a gene therapy setting) has toxicity. These however should not differ in the setting of this gene therapy study and therefore should be considered acceptable.
In summary, I believe that the acute hazards for human health are acceptable in this study. Sub-acute or late toxicity is believed to be of low to very low frequency and will be adequately monitored for in the study. In addition the issue of potential late toxicity is of lower relevance to the study population of metastatic cancer patients.

Answer 6: Minor and well represented in the documents

Answer 7: see 2.2.

3.2 Immediate and/or delayed adverse effects on the human health resulting from the potential direct or indirect interactions between the GMM and people coming in contact with the treated patient and/or with the GMM (workers, patient relatives, etc...).

Answer 1: As the maximum dose given to the patient was found to be safe and nonpathogenic, the accidental spill-over to innocent bystanders will only be a fraction of this dose and can therefore be considered harmless.

Answer 2: Simple contact with the treated patient will cause no harm. Upon intra-tumour injection, the virus can be found in the blood in low amounts. Therefore, the producers cannot exclude that virus is present in biological fluids like urine, faeces and saliva. If present, the low number of virus particles that become excreted may infect a few cells of the persons that are in contact but this will cause no problem. No new virus will be produced.

Answer 3: The vector TG1024 cannot spread in the environment due to its non replicative character (E1 deletion) and thus requires the specific cells of the laboratory, such as PER.C6 complementing cell line. This cell line has no homology between vector and helper sequences to avoid replication-competent adenoviruses (RCA) generation during adenoviral vector production. In addition, as

mentioned before, the parental wild type strain is moderately pathogen in human. Local injection of the adenoviral vector in a particular target tissue confines large amount of released vector particles to a limited area near the injection site, limiting the risk of horizontal transmission to the people (workers, patient relatives, etc...) accidentally exposed to adenoviral vectors or RCA. However, it cannot be ruled out that the recombinant adenovirus can exchange its genetic material during coinfection of the same human cell by a wild-type adenovirus and thus reacquires a replication capacity generating RCA. The probability of occurrence of this event is extremely low and would involve only a limited number of viral particles which would be rapidly eliminated by the immune system, and consequently would have no effects on health of the persons in contact with the treated patient after a putative horizontal transmission. In addition, the respect of confinement, carrying out of protection, control and monitoring measures could reduce significantly the likelihood of post-release dissemination of the vector to other persons.

Answer 4: Negligible.

Answer 5: From previous experiences this risk has been demonstrated to be absent if at the time of administration the necessary precautions are taken by the health workers at the time of preparation and administration of recombinant adenoviral vectors (this will be done by the investigators in this study). The rAd-Il2 construct under study should not be considered an exception to these previous experiences (which are well referred to by the sponsor).
Since the presence of rAd has been demonstrated in body fluids, mainly within the 24 hours following administration, it should be advised to all contacts (health care workers and patient relatives or visitors) to avoid direct contact with body fluids and secretions within the first 24 hours following administration. More stringent measures are not necessary since infectious recombinant virus will no longer be present or only at extreme low numbers, which carry no health risk.
In view of the study design (see protocol), I assume the patients will be hospitalised for at least 24 hours. During this time span contacts with body fluids and secretions should be followed by washing off and disinfections of skin contact (as described in Item 8 of this dossier).

Answer 6: Negligible. See also earlier literature with similar constructs in the past.

Answer 7: Adenoviruses are relatively stable and can potentially spread relatively easily in the environment without being inactivated. In addition, adenoviruses are typically airborne pathogens. Hence, adenoviral vectors or RCA could relatively easily be transmitted via aerosols. In addition, transmission via blood or other body fluids or perhaps excreta is possible, especially in circumstances where the vector is administered systemically or when significant vector or RCA shedding occurs in excreta, semen or saliva. Local injection of the adenoviral vectors in a particular target tissue or in a tumor, as in the present study, will likely confine gene transfer to a limited area near the injection site, limiting the risk of horizontal transmission. Nevertheless, dissemination of adenoviral vectors beyond the injection site in preclinical and clinical trials has been reported. The risk of horizontal transmission is likely to be higher in patients enrolled in clinical trials that received a high dose of adenoviral vectors by direct systemic administration than in individuals that were accidentally exposed to adenoviral vectors (or RCA). If adenoviral vector shedding occurs, it will likely be transient, unless there is continuous RCA production in vivo, which is unlikely in an immuno-competent host. Adenoviral vector particles are relatively rapidly eliminated and cleared from the circulation mainly because of the reticuloendothelial system. This significantly reduces the risk of horizontal transmission, especially following exposure to blood.

In the event that the vector would somehow find its way to a new host (via shedding, aerosols or blood transmission) the vector and/or transduced cells will likely be eliminated by the host's immune system, unless of course the new recipient is immuno-compromised. In addition, some individuals that have naturally been exposed to wild-type adenovirus, developed neutralizing antibodies which constitute an additional barrier that protects against potential horizontal transmission of adenoviral vectors.

Wild-type adenoviral infections have not been shown to cause debilitating illness in immunocompetent adults and adenoviral vaccines against Ad4 and Ad7 have an established safety record in vaccination of more than 10 million military recruits over 20 years. This strongly suggests that if inadvertent horizontal transmission were to occur with replication-deficient adenoviral vectors or even with RCA that the risks would be minimal, unless perhaps if recipients were to be exposed to an unusually high vector/RCA dose or unless the transgene itself has undesirable properties or could trigger adverse effects. This is unlikely in the present clinical study.

3.3 Immediate and/or delayed adverse effects on the environment and animal health resulting from the potential direct or indirect interactions between the GMM and other species.

Answer 1: Human adenovirus type 5 has a rather specific tropism for humans. Because nothing has been changed on the outside of the IL2-recombinant adenovirus, we do not expect a change in host target.

Answer 2: TG1024 cannot be found in the natural environment due to its non replicative character (E1 deletion) and its incapacity to propagate in the natural environment. The amount of post-release dissemination of recombinant adenoviral particles is assumed to be a fraction of the administered dose, and consequently the probability to disseminate in other mammals is extremely low. In addition, the human adenoviruses, namely HAdV-5 from which Ad-IL2 vectors are derived, is not pathogenic to animals and does not form tumors even in permissive animal models (Shenk, 2001).

Answer 3: Negligible..

Answer 4: None

Answer 5: Negligible

Answer 6:: Though mouse cells can be transduced with adenoviral vectors they are non-permissive for adenoviral replication. There are only two non-human species that allow replication of human adenoviruses, namely the cotton rat [Wilner et al, 2002] and the hamster. Human adenovirus causes pulmonary histopathology in these animal models equivalent to the corresponding human disease [Pacini et al, 1984; Hjorth et al, 1988].

3.4. Is there a significant probability that the GMM will spread from the patient to other persons or to the environment?

Answer 1: see also 4.2.

Answer 2: Adenoviruses and adenoviral vectors (or RCA) are relatively stable and can potentially spread relatively easily in the environment without being inactivated. In addition, adenoviruses are typically airborne pathogens. Hence, adenoviral vectors or RCA could relatively easily be transmitted via aerosols. In addition, transmission via blood or other biological fluids is possible, especially in circumstances where the vector is administered systemically. In the current study, IT administration confines the Ad-IL2, at least the large amount of the latter, to a limited area near the injection site, limiting the risk of horizontal transmission. Nevertheless, dissemination of Ad-IL2 vector beyond the IT injection site in several clinical trials has been already reported. Data available after the phase I trial of the current study, show the presence of viral DNA in the blood of patients, measured with specific tests (PCR), 1 to 2 hours after injections but rarely and only at low levels 24 hours after TG1024 IT injection. The TG1021 DNA was also detected in blood samples only at day 0 post IT injection and up to day 4 in patient receiving high doses. The faeces and tonsils were also positive up to day 4 and up to day 14 respectively.
The risk of horizontal transmission is likely to be higher in patients enrolled in clinical trials that received a high dose of adenoviral vector by direct systemic administration (intravenous or subcutaneous) than in individuals that were accidentally exposed to adenoviral vectors or RCA. If adenoviral vector shedding occurs, it will likely be transient. Adenoviral vector particles are relatively rapidly eliminated and cleared from the circulation mainly because of the reticuloendothelial system. This significantly reduces the risk of horizontal transmission, especially following exposure to blood. This risk may be higher however, following aerosol administration, for instance in the setting of a cystic fibrosis clinical trials which would not be the case with the IT injections of Ad-IL2.

In the event that the vector or RCA would spread from the patient to other persons (via shedding, aerosols or blood transmission) the vector, RCA and/or transduced cells will likely be eliminated by the host's immune system unless the new recipient is immuno-compromised. In addition, some individuals that have naturally been exposed to wild-type adenovirus developed neutralizing antibodies (already mentioned high seroprevalence of neutralizing antibodies to HAdV-5 in human population) which constitute an additional barrier that protects against potential horizontal transmission of adenoviral vectors or RCA. This strongly suggests that if inadvertent horizontal transmission was to occur with replication-deficient adenoviral vectors or even with RCA, the risks would be minimal.

Answer 3: The risk appears to be very low

Answer 4: No

Answer 5: No

Answer 6:: The risk of horizontal transmission to people coming into contact with the treated patient is low but it cannot be excluded. Since (infective) vector particles can be detected in excreta, the vector can wind up in the environment but since it is replication deficient it is not expected to propagate.

3.5 Are there potential risks to offspring, including vertical transmission?

Answer 1: Not from the recombinant virus itself, but maybe from the IL2 that is expressed? I have not enough experience with IL2 in order to estimate possible adverse effects of IL2 on pregnancy and/or the fetus.

Answer 2: Following somatic gene therapy with adenoviral vectors, the vector may reach the gonadal tissue where it may inadvertently transduce germ cells. However, there are several significant barriers that need to be overcome before the vector could find its way to the spermatozoids or the oocytes. These barriers include the *zona pellucida* and the cumulus mass that surrounds the oocyte and the *tunica propria* that surrounds the seminiferous tubules in the testis. These physical barriers make inadvertent germline gene transfer unlikely, at least in adult recipients. Direct injection of adenoviral vectors into either the testis or epididymis resulted in transgene expression only within the interstitium of the testis and not within seminiferous tubules. Despite direct exposure of spermatogenic cells or mature sperm to high titers of adenoviral vector, transgene expression was likewise not detected in embryos (Hall *et al.,* 2000). Similarly, there was no evidence of vertical transmission following intraprostatic administration in mice (Paielli *et al.,* 2000). In another study, the risk of insertion of adenoviral vector genomes into female germ cells during the course of somatic gene therapy was stringently tested in mice by injecting a high adenoviral vector dose into the ovary and by incubating naked oocytes with high titer adenoviral vectors (Gordon, 2001). Transgene expression was observed in the thecal portion of the ovary, with no staining seen in the oocytes. Mice with injected ovaries were mated, and preimplantation embryos or fetuses were analyzed either for transgene expression or by PCR specific for the transgene. None of 202 preimplantation embryos stained positively for the transgene and none of the 58 foetuses were positive for DNA by PCR analysis. Finally, more than 1400 eggs were fertilized after exposure to the adenoviral vector prior to *in vitro* fertilization and transgene expression was evaluated in the morulae. Fewer than 2% of the embryos stained positively for the transgene, and experiments indicated that the staining was due to incomplete washing of the eggs prior to IVF. These data provide strong evidence that adenoviral vectors cannot readily transduce oocytes and that the risk of female germ-line transduction with such vectors is very low. In addition, the biodistribution studies carried out in rat with Ad-IL2 after single intravenous injection and in monkey with Ad-IFN_ after repeated subcutaneous injection, did not show persistence of the presence of vector DNA sequences in gonads. In the monkey, no vector DNA was found in the gonads (Pecheur, 1998), while in the rat, although the vector sequences were detected in the gonads one day post injection, no sequences persistence was found in this tissue (Christ *et al.,* 2001). In the unlikely event that the adenoviral vector would somehow manage to transduce the germ cells, transduction would most likely have only short-term consequences since most adenoviral vector genomes remain episomal (Wang and Taylor, 1993) and are therefore gradually lost upon cell division during embryogenesis. Based on these preclinical studies, the risk of inadvertent germline gene transfer in patients enrolled in the further clinical trials with TG1024 is expected to be very low. The risk of inadvertent germline gene transfer resulting from accidental or occupational exposure to adenoviral vectors or even RCA, which would typically involve a much lower dose than what is required in clinical protocols, would be even lower.

Answer 3: Unlikely or again very low.

Answer 4: The measures foreseen by the protocol are adequate to eliminate any risk of this nature. Even if the patients would not follow up these instructions, the chance for vertical transmission should be considered to be extremely small to none-existing.

Answer 5: No affirmative literature with regard to this point. However, a small fraction of recombinant virus could theoretically integrate in the genome of reproductive organs. However in prior stduies this point has never been raised or considered a reason for not allowing the GMM in similar studies, also in Belgium. There are no new data that would indicate such hazard.

Answer 6: Following somatic gene therapy with adenoviral vectors, the vector may reach the gonadal tissue where it may inadvertently transduce germ cells. However, there are several significant barriers that need to be overcome before the vector could find its way to the spermatozoids or the oocytes. These barriers include the zona pellucida and the cumulus mass that surrounds the oocyte and the tunica propria that surrounds the seminiferous tubules in the testis. These physical barriers make inadvertent germline gene transfer unlikely, at least in adult recipients. Direct injection of adenoviral vectors into either the testis or epididymis resulted in transgene expression only within the interstitium of the testis and not within seminiferous tubules. Despite direct exposure of spermatogenic cells or mature sperm to high titers of adenoviral vectors, transgene expression was likewise not detected in embryos (13 Hall2000) or sperm cells (14 Blanchard 97). Similarly, there was no evidence of vertical transmission following intraprostatic administration in mice (15 Paielli 2000).

In another study, the risk of insertion of adenoviral vector genomes into female germ cells during the course of somatic gene therapy was stringently tested in mice by injecting a high adenoviral vector dose into the ovary and by incubating naked oocytes with high titer adenoviral vectors [Gordon, 2001]. Transgene expression was observed in the thecal portion of the ovary, with no staining seen in oocytes. Mice with injected ovaries were mated, and preimplantation embryos or fetuses were analyzed either for transgene expression or by PCR specific for the transgene. None of 202 preimplantation embryos stained positively for the transgene and none of 58 fetuses were positive for DNA by PCR analysis. Finally, more than 1400 eggs were fertilized after exposure to the adenoviral vector prior to in vitro fertilization and transgene expression was evaluated in the morulae. Fewer than 2% of the embryos stained positively for the transgene, and experiments indicated that the staining was due to incomplete washing of the eggs prior to IVF. These data provide strong evidence that adenoviral vectors cannot readily transduce oocytes and that the risk of female germ-line transduction with such vectors is very low. In the unlikely event that the adenoviral vector would somehow manage to transduce the germ cells, transduction would most likely have only short-term consequences since most adenoviral vector genomes remain episomal [Wang et al, 1993] and are therefore gradually lost upon cell division during embryogenesis, unless integrating adenoviral vectors are employed. Based on these preclinical studies, the risk of inadvertent germline gene transfer in patients enrolled in clinical trials is expected to be very low. The risk of inadvertent germline gene transfer resulting from accidental or occupational exposure to adenoviral vectors or even RCA would be even lower, which would typically involve a much lower dose then what is required in clinical protocols.

3.6. Are there any selective advantages or disadvantages conferred to the GMM compared to the parental organism?

Answer 1: Since the GMM is a non-replicative and incomplete virus, there is absolutely no selective advantage in terms of spreading, survival or pathogenicity as compared to the wild type
Answer 2: Not relevant. It does not replicate/spread.

Answer 3: As already described, Ad-IL2 is issued from the wild type of HAdV-5 deleted in E1 and E3 genes and thus replication-defective (desirable disadvantage to GGM compared to the parental organism). Obtained vector conserves all other characteristic of the parental virus (there are no advantages compared to the wild type of HAdV-5). Since E1 gene products are indispensable for viral multiplication E1 deleted recombinant adenovirus can be produced only in a cell line which expresses E1 proteins.

Answer 4: The GMM has a selective disadvantage for production and spread compared to the parental organism because it has been rendered replication incompetent. However, there is always the low probability that complementation can occur with a wild-type virus and restore replication competence.

Answer 5: Yes, the deletion within the recombinant adenovirus eliminates his capacity to replicate without the complementation of the producer cell line (the information provided by the sponsor regarding this topic is complete and adequate). When complemented by replicating wild type virus, the recombinant is disadvantaged and patient immune reaction will neutralise any replicating form (wild type or recombinant).

Answer 6: The advantage is that it can not replicate and that it contains a therapeutic gene.

Answer 7:: The Ad-CMV-IL-2 vector is not expected to have any selective advantages over the wt adenovirus, on the contrary, since it is replication-deficient and expresses a gene that stimulates the immune systems it is more likely to have a selective disadvantage compared to the parental organism.

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

Answer 1: Impossible

Answer 2: The generation of contaminating E1-positive RCA is a major safety concern associated with the production of adenoviral vectors. The presence of RCA in batches that are to be used for clinical trials is undesirable, as it may induce significant pathological side-effects. The presence of RCA is associated with inflammatory responses (Hermens and Verhaagen, 1997). These inflammatory responses may be caused by the tissue damage that is triggered by the adenoviral replication. Alternatively, the production of susbstantial amounts of potentially immunogenic and toxic adenoviral proteins could worsen the inflammation. RCA may replicate in an uncontrolled manner in the patient, especially in immunocompromised recipients. In normal individuals, the immune response is believed to limit viral spread. In addition, some individuals that have naturally been exposed to wild-type adenovirus developed neutralizing antibodies which constitute an additional barrier that protects against potential horizontal transmission of adenoviral vectors or RCA. However, in infants with an intact immune system, adenovirus infection, can cause severe health problems and even death (Munoz *et al.*, 1998). In addition, RCA could act as a helper to mobilize or rescue E1-deleted adenoviral vectors in vivo, potentially increasing the effective dose of vector as well as its mobilization throughout the recipient.

The generation of RCA is primarily due to homologous recombination between adenoviral vector and helper sequences in the packaging cells, particularly in the commonly used 293 cells and other cell lines such as 911 cells (Fallaux, 1996). When typical E1-deleted adenoviral vectors are propagated in 293 cells, there is significant sequence homology between vector and helper sequences of up to 450 bp at the left side of the transgene and approximately 800 bp at the right side, that results in the formation of RCA by homologous recombination. Theoretically, one recombination event in the region downstream of the transgene would have been sufficient to generate RCA since the entire left end of the Ad5 genome is present in 293 cells. Nevertheless, all of the studied RCA had undergone a double recombination event, upstream and downstream of the transgene, resulting in the replacement of the transgene with the E1 gene (Lochmuller *et al.*, 1994; Hehir, 1996). All known RCA are similar

to wild-type adenovirus except that in most cases the E3 region is deleted in accordance with the configuration of the vector.

To avoid RCA generation during adenoviral vector production the sequence homology between vector and packaging cell line should be eliminated. Cell lines have now been developed with reduced homology or no homology at all between vector and helper sequences, such as the PER.C6 and A549 cell lines (Imler *et al.*, 1996; Fallaux *et al.*, 1998; Fallaux *et al.*, 1999). The combination of PER.C6 cells and matched vectors that do not share any homologous sequence eliminates the generation of RCA by homologous recombination. Alternatively, RCA generation could be reduced by relocating or deleting the pIX gene (Hehir *et al.*, 1996). The use of these improved packaging cells for RCA-free adenoviral vector production is critically important for the production of clinical grade vector batches but is also warranted for use in the laboratory and manipulation in animals given their improved safety profile. Various tests have been developed, including rescue assays and PCR-based approaches that can be used to screen vector batches for potential RCA-contamination (Dion *et al.*, 1996; Nehir *et al.,* 1996; Fallaux *et al.*, 1998).

The vector ADTG13383 used in the current study was generated and propagated in PER.C6 complementing cell line to avoid any occurrence of RCA. The presence of RCA in batches destined to be used for clinical trials was tested by inoculation of human cell line A549 and RCA were not detected in each clinical lot used in clinical study.

Answer 3: It would not be able to reconvert to the wild-type form without complementation (see below)

Answer 4: This possibility is extremely remote and unlikely to occur in view of the replicative disadvantage of such forms.

Answer 5: No

Answer 6:: The Ad-CMV-IL-2 could reconvert to its wild-type form by homologous recombinant with wild-type Ad. This would require that the patient would be naturally exposed to wild-type Ad and there does not appear to be an added risk of generating wt Ad from this recombination event, compared to the inherent consequences of a natural Ad infection.

3.8 What is the possibility of the GMM to be complemented in vivo by replicating competent viruses and what are the possible consequences for the human health and environment?

Answer 1: (Van Tendeloo): The risk for genetic recombination requires co-infection of the transduced cell with a wild type Ad5 virus and hence this risk is very low. If it should happen, the recombination will not extend beyond the classical viremia of Ad5.

Answer 2: The producers of this recombinant product admit that the virus that has been injected at high titers in the tumour can come into the blood and may be spread to different organs, including lungs. This means that it is theoretically possible that recombinant virus gets into a cell that is infected with replicating competent viruses. However, this chance is very low. Replication of wild type virus takes place during a short period of one to two weeks and especially during childhood. The chance that this coincides with the injection is then of course very low. Further, what is the effect of recombinations that may occur? Only handicapped viruses will come out that do not replicate (E1 region/suicide-recombinants) or that do not show immune evasion (E3 region). It can be expected that the latter will normally be eliminated effectively by the raised immunity.

Answer 3: Exposure of patients enrolled in clinical trials to RCA or to naturally occurring wild-type adenovirus could mobilize E1-deleted adenoviral vectors *in vivo*, which would increase the risk of horizontal transmission of the vector. Also, transcomplementation of adenoviral vector *in vivo* could induce potent inflammatory reactions and contribute to toxicity. However, the extent of vector mobilization by wild-type virus or RCA may be relatively limited at least when one bases oneself on animal models permissive for adenoviral replication. Oualikene et al. (1995) reported that there was no evidence of phenotypic complementation *in vivo* of E1-deleted HAdV-5 upon superinfection by wild-type HAdV-5 in the cotton rat. This species is permissive for HAdV-5 replication, and therefore it is a good model for the *in vivo* study of biosafety of adenovirus-mediated gene therapy in human beings (Pacini *et al.,* 1984). This suggests that the results obtained in the cotton rat model could be extended to patients treated with an E1 deleted HAdV-5 based vector in case of superinfection with a wild type of HAdV-5. Although, such an event is not an impossible occurrence, its frequency, and consequently the risk of phenotypic complementation in the study where the patients are treated with Ad-IL2 injected by intra tumoral route, must be very low and this event is not likely to cause any significant harm in patients for the following reasons: a) the phenotypic complementation will not extend beyond viremia of HAdV-5, which is known to be relatively rapidly eliminated and cleared from the circulation, so the vector will be transcomplemented as long as the wild type parental virus propagates; b) this transcomplementation will be limited to the tissue were both type of viral particles (wild type and E1 deleted) are numerous enough to simultaneously infect the same cell, and consequently, only a fraction of the dose of administered vector will be amplified, but less efficiently than the amplification of the wild type virus; c) parental adenoviruses do not propagate naturally in the place like site of injection, and therefore a putative transcomplementation will occur on vector particles that have disseminated from this site which is a small amount of the administered dose; d) batches used in clinical trials are free of RCA.

Answer 4: If the GMM is complemented with a wild-type virus and gains replication competence, then the risks are the same as for infection with the wild-type virus. However, if there is partial recombination, the risks are unknown. Could a new adenovirus be produced that becomes replication competent and expresses the IL-2 gene? Could an adenovirus containing the CMV promoter be expressed at higher levels and therefore gain increased pathogenicity? These scenarios seem to be of very low probability, but not impossible.

Answer 5: There is a remote possibility for the recombinant to be complemented and replicate during adenovirus infection. However, the recombinant will be disadvantaged for replication. In addition, neutralising host immunity that is boosted by wild type infection will also neutralise the recombinant. As such the possible consequences are of extreme low probability.

Answer 6: The complementation would be very improbable and with limited consequences. This issue is addressed adequately in the documents However, although theoretically the genome of the recombinant virus can not recombine with the genome of wildtype parent adenovirus based on the sequence comparison of both, this possibility has not been investigated preclinically as can be ascertained from the documents. Nature does not always follow the theory. Therefore this remains a relevant question with regard to public health. In earlier dossiers with similar adeno-derived GGM this was done (with no recombinations observed). So my question is whether such preclinical testing should be done. In the current study this problem is partially tackled by looking for the occurrence of RCA in patients treated with the GGM.

Answer 7: Exposure of patients enrolled in clinical trials to RCA or to naturally occurring wild-type adenovirus could mobilize E1-deleted adenoviral vectors in vivo, which would increase the risk of horizontal transmission of the vector. Other viruses such as HPV or EBV may also trigger adenoviral replication of E1-deleted vectors and increase the risk of horizontal transmission. However, the extent of vector mobilization by wild-type virus or RCA may be relatively limited at least based on animal models permissive for adenoviral replication [Imler et al, 1995]. The expression of IL-2 may further decrease vector spread and shedding due to enhance anti-viral immune responses

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

Answer 1: The tropism of recombinant Ad is limited to eukaryotic cells bearing the Coxsackie and adenovirus (CAR) receptor which bind the Ad pentons of the viral coat.

Answer 2: Exchanges between genetically different micro-organisms will normally not occur.

Answer 3: At present, there is no evidence, to my knowledge, that adenoviral vectors could transfer a transgene to other micro-organisms. However, this cannot be completely excluded.

Answer 4: There is a possibility of recombination with other wild-type adenoviruses, but then again this risk is very low due their probability of being in the same cell at the same time.

Answer 5: Also, such a possibility is to be considered of extreme low probability.

Answer 6: No

Answer 7: unknown

4. Questions related to the risk assessment of GMM

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 GMM?

Answer 1: The magnitude of the potential risks of the GMM for the patient are low when the injected dose will not exceed the MTD as established in their previous clinical study

Answer 2: The strain TG1024 (E1 and E3 deleted) is non replicative on normal cells, but only on cells that transcomplement the missed functions, and therefore cannot be found in the natural environment and also is no longer able to cause disease as the wild type of HAdV-5. The probability that the missing E1 function is complemented is extremely low in natural ecosystem. In conclusion, the magnitude of identified potential risks, described hereinbefore, related to the GMM, is low.

Answer 3: Unfortunately, I do not really understand what you are getting at with these two questions.

Answer 4: See answer to 4.1

Answer 5: Minor and verifiable

Answer 6: The risk of horizontal and vertical transmission and the risk to the environment related to the Ad-CMV-IL2 vector is likely very low. However, the risk to the patient is much higher given several unknowns related to the non-linear threshold effect, inter-patient variation, inter-species differences, adverse effects of DTIC, IL-2 and Ad combined and the lack of data on the preclinical safety evaluation of Ad-CMV-IL2 +/- DTIC in non-human primates.

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

Answer 1: No risk for human health and environment

Answer 2: 5.1./5.2. Extremely low (difficult to give it a precise weight).

Answer 3: Human adenoviruses belong to Risk Group 2 (Directive 2000/54EC of 18 September 2000). This group includes the pathogens associated with diseases that are rarely serious and for which preventive/therapeutic interventions are often available. Laboratory exposures rarely cause infection leading to serious disease and the risk of spread is limited, particularly since the GMM (TG1024) is E1 deleted and thus replication incompetent. Consequently, potential risks related to the GMM are then categorized in class 2.

Answer 4: Unfortunately, I do not really understand what you are getting at with these two questions.

Answer 5: See answer to 4.1

Answer 6: Minor and verifiable

Answer 7:: see 5.1

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: Yes

Answer 2: Based on data obtained from the patients in the previous experiences with TG1021 and TG1022 as well as in the phase I trial conducted I trial conducted (Switzerland) in patients with TG1024, I think that the monitoring plan proposed by the applicant could confirm the validity of the

hypothesises issued during the risks evaluation concerning the potential adverse effects and could allow to identify and manage the occurrence of eventually non-anticipated adverse effects.

Answer 3: Yes

Answer 4: Yes

Answer 5:: The monitoring plan proposed by the applicant addresses the risks concerning potential adverse effects, in particular by evaluating the risk of horizontal and vertical transmission using PCR-based techniques in blood, tonsils, urine feces from the human patients infected with the GMO. The safety with respect to the patient, particularly with respect to the known risks of adenoviral gene transfer will be monitored, in particular thrombocytopenia, liver toxicity, inflammatory responses, IL-6, DIC will be closely monitored in addition to standard toxicological analysis and clinical observation immediately prior to, during and for 4 hrs after injection, which should also allow for the identification of non-anticipated acute adverse effects in the patient.

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

Answer 1 In my opinion, all necessary measures are taken and they are sufficient.

Answer 2: None

Answer 3: None

Answer 4: No additional complementary measures regarding the monitoring of the risk of horizontal and vertical transmission seem to be required.

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

Answer 1: No real biosafety measures will be taken because there is no danger of spread of the GMM.

Answer 2: Manipulation of the recombinant adenovirus is conducted in a L2 laboratory environment which means that all measures are taken in compliance with requirements for risk level 2. In addition, the vector is propagated on PER.C6 cells that do not share any homologous sequence with the former. This enables to avoid any occurrence of RCA. Also, the presence of RCA in batches destined to be used for clinical trials was tested by inoculation of human cell line A549 and RCA were not detected in each clinical lot used in clinical study.

Patients will be treated and stay in a single hospital room (containment level 2) during the observation period. Blood samples will be taken from patients before and then 1 and 2 hours after the first two injections and 24 h after the first injection in patient cohorts 1 to 7 and also urines, faeces and tonsils from patients belonging to cohorts 8 and 9 in order to monitor potential viral spread. This will be analysed by polymerase chain reaction (PCR) (search for the presence of viral DNA) with the option of culture (search for infectious viral particles). Contact between patients will be avoided and their wastes will be treated according to hospital specific rules for infectious waste. Access to the area of clinical trials is restricted only to personnel participating in the clinical trial, physicians, nurses, and pharmacist. They will be informed in detail of the objectives of the clinical trial and its protocol, as well as the nature of the product to be handled, any risks related to the product, handling procedures to

follow and steps to take in case of an accidental dispersion of the product according to emergency plans. During product manipulations goggles and lab coat must be worn, gloves are recommended. Prior to administration, the final product (TG1024) must be prepared under conditions compliant with requirements for injectable preparations.

All materials used for preparation and administration will be placed in closed containers and then decontaminated according to the regular hospital procedures for infectious material or according to the disinfection protocol for therapeutic units by Transgene S.A. Decontaminated wastes will be then discarded according to the regular way of destruction for hospital wastes. Appropriate antiviral product will be regularly used as a viral disinfectant at the hospital.

Answer 3: See item 8 of this dossier. These measures are adequate and sufficient.

Answer 4: Sufficient

Answer 5:: The GMO is released only for clinical use according to standard Good Clinical Practice conditions. Patients are kept in individual rooms to improve containment.

5.4 If you have identified potential risks to offspring, which complementary measures could be considered to minimise the risk? Should birth control measures be recommended to patients?

Answer 1: As previously described (see response to question 4.5.), the risk of inadvertent germline gene transfer in patients enrolled in the further clinical trials with TG1024, is expected to be extremely low, if negligible. In addition, the women enrolled in clinical trials must have a negative pregnancy test at entry and adequate protection against pregnancy is required during the conduct of the study for patients (male and female).

Answer 2: Even in the absence of birth control measures the risk for vertical transmission is limited in this study population. In addition adequate recommendations for birth control while on study are made to the patients.

Answer 3: Birth control for several months would be reasonable (has been applied
to earlier protocols with similar constructs

Answer 4: Although the risk of vertical transmission is extremely low, it would be prudent still to take birth control measures to further decrease this risk.

5.5. Which type of waste could be generated?

Answer 1: Empty ampoules, syringes and needles. These should be autoclaved.

Answer 2: Laboratory biohazardous waste, such as discarded cultures, tissue, media, plastics, and other materials, generated in UTCM (Unité de Thérapie Cellulaire et Moléculaire) of Transgene S.A. during the production of the vector used in clinical trials, were decontaminated by autoclaving before disposal. Waste collected in leak-proof containers was then incinerated.

Waste that could be generated in post-release treatment, precisely, after preparation and intra tumoral administration of the vector in hospital, is composed of opened ampoules, tubes for dilution, syringes, needles, gloves, gauze dressing. This waste is placed in closed containers treated according to regular hospital procedure for infectious material. Lab coats, goggles, patient gown, bedding, are

decontaminated according to the regular hospital procedure for infectious.

Answer 3: This item is covered by item 8 of the dossier.

Answer 4:: The waste treatment proposed by the applicant is satisfactory.

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

Answer 1: All material should be autoclaved.

Answer 2: I think that the waste treatment proposed by the applicant is sufficient.

Answer 3: It is satisfactory.

Answer 4: Yes

Answer 5:: The waste treatment proposed by the applicant is satisfactory.

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

Answer 1: For broken ampoules, contacts with the skin and wounds, it would be the best to propose desinfectants which have been proven to be effective against adenoviruses.

Answer 2: No matter how carefully one works, laboratory accidents occur and necessitate emergency response. I think that emergency plans tailored by applicant for a biohazardous situation in the current study (risk class 2), should assure the control of the potential negative effects.

Answer 3: Yes

Answer 4:: The emergency plans are adequate.

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

Answer 1: In my opinion, complementary measures could be superfluous.

Answer 2: None

Answer 4:: The emergency plans are adequate.

5.9. 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: It would be advisable to reduce the person contacts of the patient to a minimum (no visit, especially with naive children) during a period of two weeks after the first injection, in order to

exclude the possibility of the spread of a recombinant virus, that theoretically may be formed (see 4.8.), to another person. During this period, we expect an appropriate immunological response shortly after the first injection that will eliminate eventually formed recombinant virus.

Answer 2: Relative ease of making use of measures to control the risk proposed by the applicant allow reducing the potential negative effects with. Nevertheless, additional biosafety measures (others than the test of the vector batches on A549 cells to detect eventual generation of RCA), could be also applied, such as, for instance, safety validation and assessment of potential replication-competent adenovirus contamination and vector complementation *in vivo* when performed in animal models that allow replication of HAdV-5, namely the cotton rat and the hamster. These species are permissive for HAdV-5 replication and therefore it is a good model for the *in vivo* study of biosafety of adenovirus-mediated gene therapy in human beings (Pacini *et al.,* 1984). This suggests that the results obtained in the cotton rat model could be extended to patients treated with an E1 deleted HAdV-5 based vector in case of superinfection with a wild type of HAdV-5. However, it was already reported that there was no evidence of phenotypic complementation *in vivo* of E1-deleted HAdV-5 administered intravenously or intramuscularly upon intranasal superinfection by wild-type HAdV-5 in the cotton rat (Oualikene *et al.,* 1995).

Answer 3: They are sufficient.

Answer 4: Risks are already minimal. Control measures , in casu monitoring RCA, are Sufficient

Answer 5: The measures to control the risk according to GCP, the waste disposal and standard decontamination procedures and the emergency plans allow to reduce the potential negative effects .

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: Yes, my expertise does not cover the topics of question 5.

Answer 2: potential risk of IL2 for the pregnant uterus/fetus (question 3.5)

Answer 3: In my opinion, an advice on the questions related to the monitoring, waste and emergency plans proposed by the applicant (5.1.- 5.9.) could be also sough from other expert who has experience in this domain.

Answer 4: I simply do not understand 4.1-4.2 and do not feel I have the expertise to fully answer 5.1-5.9

Answer 5: No.

Answer 6: questions 5.6, 5.7 and 5.8

Answer 7: we are unaware of the actual required detection limits of the different assays used in QC testing

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