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**Report from the
Ad hoc meeting of CPMP Gene Therapy Expert Group
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Chairperson: K. Cichutek

Introduction

The “Ad hoc Gene Therapy Expert Group“ (GTEG) of the Committee for Proprietary Medicinal Products (CPMP) may identify areas in the existing “Note for guidance on the quality, preclinical and clinical aspects of gene transfer medicinal products (CPMP/BWP/3088/99)“ which require up-dating or strengthening by developing appropriate supplementary explanatory notes or position papers, to ensure that the regulatory guidance is kept current and reflects the current state of play. The GTEG convened on January 23-24, 2003, to discuss:

- A. New information on insertional oncogenesis, based on the analyses of available non-clinical and clinical data following the two serious adverse events (SAEs) recently reported in a gene therapy trial carried out in France for the treatment of children affected by X-linked severe combined immunodeficiency (X-SCID).
- B. A survey of published non-clinical testing studies on potential inadvertent germline alteration using retroviral, AAV-derived and adenoviral vectors as well as plasmid DNA, each encompassing a specific expression vector. A proposal for the review of current recommendations was also considered.

Scientific Discussion

A. Insertional oncogenesis: non-clinical and clinical models

The GTEG discussed the safety issues generated by the SAEs in the French X-SCID gene therapy trial. It received updates from the invited experts on the general risk of insertional oncogenesis with retroviral vectors, on the clinical and laboratory findings on the two patients that developed the SAEs, and on the available data from the X-SCID gene therapy clinical trial carried out in the U.K.

The rare genetic disorder X-SCID is caused by mutations in the common γ_c -chain (γ_c) gene, encoding a common subunit of receptors for cytokines that give proliferation or anti-apoptotic signals to lymphocytes (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21). The disease is characterized by a lack of B and T lymphocytes, and severe and recurrent infections that are usually fatal in the first years of life. Bone marrow transplantation (BMT) from a related, HLA-compatible donor is the treatment of choice, although it is available for less than one third of the X-SCID patients. For the others, unmatched BMT carries a high risk of graft failure, graft-versus-host disease, and lymphoma. The gene therapy treatment developed at the Hôpital Necker Enfants Malades in Paris involves autologous transplantation of bone marrow cells including CD34⁺ hematopoietic stem/progenitor cells genetically modified ex vivo by a retroviral vector carrying a copy of the γ_c gene under the transcriptional control of the enhancer/promoter elements contained in the vector long terminal repeats (LTRs).

7 Westferry Circus, Canary Wharf, London, E14 4HB, UK

Tel. (44-20) 74 18 84 00 Fax (44-20) 74 18 84 16

E-mail: mail@emea.eu.int <http://www.emea.eu.int>

The French investigators reported that this treatment has achieved effective and life-saving immune reconstitution in 9 out of 11 patients treated so far. However, two patients developed uncontrolled lymphoproliferative disorders three years after treatment, and underwent chemotherapy. The causes of these disorders have not been conclusively established, although the available data clearly point to the integration of the gene transfer vector into a sensitive region of the genome as a possible factor in the initiation of the malignant progression. As a consequence of the occurrence of those serious adverse events, regulatory agencies in Europe and the U.S. have reacted by putting on temporary hold some or all of the clinical trials involving the use of retroviral vectors.

1. The risk of retroviral vector insertion

The Expert group heard presentations and update on the theoretical risks associated with the random integration of retroviral vectors into the genome of human cells. The application of potentially genotoxic agents for the treatment of severe diseases is not unusual in medicine, and typically follows a risk-benefit evaluation. This also applies to the use of integrating gene transfer vectors, which by definition introduce mutations into the genome of treated cells. Virus-mediated mutagenesis is a known risk factor for tumor development in animals and human subjects. Accordingly, this risk has always been anticipated to be relevant for human gene therapy. However, the frequency of SAEs related to gene vector-mediated genotoxicity was difficult to predict and suggested to be highly context-dependent.

When introducing therapeutic genes (transgene) for therapeutic purposes at a predictable copy number into cells with high proliferative potential, transgene insertion into chromosomes must occur. Currently, only replication-defective retroviral (including lentiviral) vectors allow stable gene transfer into transplantable hematopoietic cells with predictable efficiency. The genetic risk associated with the chromosomal insertion of retroviruses and derived gene transfer vectors has been investigated in great detail at a pre-clinical level for more than a decade. A preintegration complex composed of the retroviral integrase, additional retroviral and cellular proteins, and the retroviral double-stranded DNA is formed shortly after cell entry. If this complex gains access to chromosomes, retroviral transgene insertion occurs with high efficiency but without clear preference for specific target sequences or loci. Therefore, each transduced cell carries a clonal genetic marker related to the retroviral insertion site. Open chromatin associated with transcriptionally active regions seems to be the preferred target of the retroviral preintegration complex. The distribution of insertion sites may thus be related to the differentiation status of the cell.

When using replication-defective retroviral vectors, the genotoxic risk of insertional mutagenesis is limited to the cells that are initially exposed to the vector preparation. Due to the untargeted distribution of insertion sites, the risk increases with the number of cells modified and with the number of insertions per cell. When using appropriate experimental conditions, the number of retroviral insertions can be limited to one per cell. Still, transgene insertion may activate or inactivate cellular genes. Special concerns are raised when the cellular gene involved may act as an oncogene or a tumor-suppressor gene.

Monoallelic gene disruption due to insertional mutagenesis usually produces a recessive mutation that requires an independent mutation in the second allele to become phenotypically relevant. Although insertional mutagenesis typically introduces fewer mutations per single cell than chemical or irradiation mutagenesis, it is associated with a higher risk of deregulation (particularly activation) of an affected locus. If the gene transfer vector carries a transcriptional enhancer, this can interfere with the expression of a genomic locus from significant distances (as much as 90 kilobases [kb]). However, it is possible that target gene deregulation occurs only from a subset of insertion sites within such a potential zone of influence. In addition, direct promoter insertion or inappropriate RNA processing may occur as a consequence of retroviral insertion.

Monoallelic transgene insertion leading to a proto-oncogene activation can be expected to result in a dominant mutation that does not require mutation of the second allele before it may contribute to cancer progression.

Assuming that the vulnerable region for activation of a particular allele is approximately 10 kb out of a total potentially accessible genome size of 1 million kb, such allele could become

deregulated with a frequency of 1 in 100,000 insertions. Since there is an estimated number of >200 cellular proto-oncogenes in the human genome, the risk of introducing a foreign gene within a critical distance from such genes may become as high as 10^{-2} . This theoretical frequency is several orders of magnitudes higher than the transformation frequency of $\sim 10^{-7}$ predicted by earlier experiments. However, at least four biological filters are expected to prevent a cell with an insertional oncogene activation to mediate the development of a clinically relevant malignancy:

- a. The low seeding efficiency (actual engraftment into the patient) after ex vivo manipulation may limit the number of potentially dangerous integration events.
- b. The oncogene-related alterations may either not affect the homeostasis of the transplanted cells (any given oncogene may promote malignant transformation of only a limited subset of cells) or induce extinction rather than expansion of the affected clone.
- c. A single genetic alteration is usually not sufficient for human cancer progression, and additional genetic events (mutations) are required to lead to uncontrolled clonal proliferation of the affected cell.
- d. Transformed cells can be eliminated by immune control or other mechanisms.

2. The French X-SCID gene therapy trial

The Expert Group was given an updated report of the French X-SCID gene therapy trial and of the available laboratory findings on the two SAEs. The trial carried out by Fischer and co-workers involved 11 patients (ten in France, one in Australia) treated at an age of 1 month to 15 years. Eligibility criteria were the presence of a mutation in the γ_c gene and the absence of a compatible bone marrow donor in the family. The protocol involved bone marrow harvesting under general anesthesia, selection of CD34⁺ cells by immunomagnetic beads, pre-activation for 24 hours by a cocktail of cytokines (stem cell factor, FLT3 ligand, TPO and IL-3) and three rounds of infection in bags precoated with retronectin[®] with a preparation of a retroviral vector (MFG) carrying a γ_c cDNA under the control of the viral LTR. The cells were re-injected into the patient's circulation after five days of culture. Pre-clinical studies in a mouse model of the disease had shown that restoration of γ_c expression by retroviral gene transfer results in rapid recovery of lymphocyte development and restoration of immune functions. The use of the viral LTR enhancer/promoter was required to restore sufficient levels of γ_c expression. The clinical study showed that gene therapy achieved effective and life-saving immune reconstitution in 10 out of 11 patients (in one patient the genetically modified cells did not engraft). Genetically corrected T cells met a strong selective advantage in vivo, providing polyclonal immune reconstitution with normal T cell counts over prolonged periods of time (>3 years for the first patient). All patients are alive, have been able to lead a normal life, and should be considered cured by the gene therapy treatment for the observation period given.

Recently, two of the ten successfully treated children developed, approximately three years after treatment, a leukemia-like lymphoproliferative disease. The clinical and laboratory data generated on the two cases were presented and discussed. In both cases children were transplanted at a very young age (1-3 months) with high doses of transduced hematopoietic progenitor cells (over 10^6 per kg). Genetic analysis showed that in both cases the malignant cells carried a proviral integration within or near the *LMO2* gene on chromosome 11p13. This gene is involved in chromosomal translocations - t(11;14)(p13;q11) or (7;11)(q35;p13) - frequently associated with childhood acute T-lymphoblastic leukemia (T-ALL). The *LMO2* locus extends 10 kb upstream of the first exon, in a region containing the promoter and regulatory sequences. Other putative regulatory elements are contained in the first intron. *LMO2* is normally expressed in CD34⁺ cells, and is down regulated in T cell development. In mice, overexpression of a *LMO2* transgene during T cell development results in clonal T cell tumors with a latency of approximately one year. In children affected by *LMO2*-related T-ALL, malignant clones carry unrearranged T-cell receptor genes, indicating that transformation occurs during thymic T cell development and before completion of T cell maturation.

2.1. The first SAE: patient #4

The first SAE was observed in the fourth patient, a child treated at the age of 1 month who developed a monoclonal $\gamma\delta$ T-cell lymphoproliferative disorder. The investigators recognized early signs of the disease (clonal expansion of $V\gamma_9/V\delta_1$ T cells) 30 months after treatment, followed its progression, and started a chemotherapy in early September 2002, when the peripheral blood T cell count reached 300,000/ μ l, and splenomegaly developed. The patient responded to the therapy with complete clinical remission. In the leukemic cells, the retroviral vector was found inserted in the first intron of *LMO2* in a reverse orientation, leading to high, constitutive expression of the affected *LMO2* allele. The γ_c transgene had a normal sequence, and was constitutively expressed, although at lower levels compared to the cellular γ_c gene in normal T cells. There was no evidence of abnormal signaling from the γ_c transgene in the malignant cells. Among factors mentioned a history of childhood tumors in the patient's family, and an episode of chickenpox infection preceding the occurrence of the malignancy that might have caused an abnormal proliferative expansion of immunoreactive T-cells. The leukemic cells were positive to a molecular test for the presence of the chickenpox (varicella-zoster) virus belonging to the α -herpes virus family. Analysis of the transduced T cells showed that the malignant clone was detected a number of months after treatment. At later stages, all malignant T cells carried an additional genetic abnormality, a t(6;13) chromosomal translocation. After the first round of chemotherapy, T cells were found that retained the proviral integration in *LMO2* but had lost the chromosomal translocation, indicating that the latter had probably occurred during malignant progression of the original *LMO2*-expressing clone. The patient is alive, and will receive a bone marrow transplantation in the near future, since an unrelated, HLA-matched donor was found in the meantime.

2.2. The second SAE: patient #5

The second SAE was observed in the fifth patient, a 3-year-old child treated at the age of 3 months, who developed an uncontrolled $\alpha\beta$ T-cell lymphoproliferative disorder. This patient received the highest cell dose in the trial, i.e., 20×10^6 cells per kg. The investigators recognized the disease in the month of December 2002, three months after the last control that showed normal blood parameters and polyclonal T-cell reconstitution. The malignant cells rose up to a count of 188,000/ μ l, with splenomegaly and mediastinal lympho-adenoproliferation. Unlike patient #4, this subject did not experience intercurrent infections, and is not known to have a family history that would be predisposing to cancer. Analysis of the leukemic cells showed again a retroviral insertion in the *LMO2* locus, upstream from the promoter and in forward orientation. Similar to the first case, the *LMO2* gene as well as the γ_c transgene were constitutively expressed in the leukemic cells. Gene expression studies and karyotype analyses of the malignant cells revealed additional abnormalities. Preliminary data indicated that the malignant T cell pool was made of three different T cell clones, all carrying the same *LMO2* retroviral insertion. The data suggest a specific pathogenesis in which *LMO2* has found a distinct set of collaborating oncogenes during the pre-leukemic expansion of the clone. The constitutively expressed γ_c transgene, which confers a selective proliferative advantage to the transduced T-cells upon engraftment, might itself have played a unique role in favoring expansion of *LMO2*-expressing clones.

3. The U.K. X-SCID gene therapy trial

An updated report of the X-SCID gene therapy trial carried out at the Institute for Child Health in London was presented. Five children were treated so far, at ages ranging from 4 months to 20 years. The cell dose administered to the patient ranged from 8 to 22×10^6 per kg. The older patient (patient #4) did not benefit from the procedure due to graft failure. The gene transfer protocol differed in several aspects from the one used in Paris: the retroviral vector was pseudotyped with a different envelope (GALV vs MLV-4070A), and the $CD34^+$ cells were cultured in a serum-free medium with a lower concentration of IL-3. This protocol seemed to lead to improved B cell reconstitution and higher marking in myeloid cells. The follow-up ranges from 18 months for the first patient to 1 month for the last one. No adverse event has been observed so far.

It was also mentioned that a clinical gene therapy trial had started for chronic granulomatous disease (CGD), a rare genetic disease due to mutations, e.g., in the *gp91-phox* gene, encoding a subunit of the phagocyte-specific NADPH oxidase enzyme complex. One patient has been treated so far. The transduction procedure involved the same cells (CD34⁺ stem/progenitor cells) and a similar transduction protocol, yet a vector encoding a different transgene (*gp91-phox*), which does not provide a selective advantage to myeloid or lymphoid cells during reconstitution. The follow-up of this patient showed no detectable vector-positive peripheral T cells, and an oligoclonal pattern of integration in B and myeloid cells.

4. The experience of the ADA-deficient SCID gene therapy trials

Data were reported from the gene therapy trial carried out at the Children's Hospital in Los Angeles (CA, USA) on a different but related disease, the SCID due to a deficiency of the enzyme adenosine deaminase (ADA-deficient SCID). Peripheral blood cells from one child who received in 1993 a transplantation of CD34⁺ cells from umbilical cord blood transduced with a retroviral vector expressing the ADA cDNA were analyzed. ADA is not a growth factor receptor, so in this case the genetically modified cells do not have an obvious growth advantage during engraftment. The patient was maintained on enzyme replacement therapy with conjugated bovine enzyme (PEG-ADA) throughout the trial, and there was a gene marking in peripheral blood of 1-10%. So far, no integration near or within *LMO2* could be found in the transduced cells. During the discussion, it was mentioned that similar data were observed in the ADA-deficient SCID gene therapy trial that started in 1992 at the San Raffaele Hospital in Milano (I). The last four patients in this trial received a partial myeloablation regimen to favor engraftment of CD34⁺ cells, and showed polyclonal reconstitution of the T, B and NK cell compartments, with no sign of T cell clonal expansion in a follow up of three months to two years.

5. Special risk factors associated to the X-SCID gene therapy trial

The occurrence of leukemic complications in two out of ten successfully treated patients in less than a year is a clear sign that the gene therapy treatment for X-SCID, as it was originally designed, involves an unforeseen and unexpectedly high risk of cancer. The repeated involvement of the *LMO2* gene as a site of insertion in the proliferating T-cell clones strongly suggests that the abnormal expression of this gene constitutes a high risk in the context of this treatment. Pre-clinical studies of the gene therapy approach used in the X-SCID study had shown no evidence of leukemia or other forms of cancer, and no similar adverse events have been reported in other gene therapy trials involving the use of retroviral vectors, including those addressing the ADA-deficient SCID, which were initiated in the U.S. and Europe more than a decade ago. Other factors could have potentially contributed to increase the risk of malignant transformation in this specific disease, including the constitutive expression of the therapeutic transgene, the young age of the patients, their genetic background, and the cell dose administered. These factors were extensively discussed by the GTEG.

5.1. Deregulated expression of the γ_c transgene

The survival signal provided by transgenic expression of γ_c in lymphoid progenitors and mature T cells of X-SCID patients allows a rapid clonal expansion that by itself elevates the risk of accumulating subsequent oncogenic mutations. Moreover, there may be weak side effects of γ_c expressed under the control of a retroviral promoter, or a related strong constitutive promoter. Although there is no evidence for increased surface expression of γ_c in the patients following gene therapy, new concerns have to be addressed in retrospect. These include the potential for insufficient down regulation of transgenic γ_c following stimulation by T cell growth factors, interference of potentially supra-physiological cytoplasmic γ_c expression with interleukin receptor cross talk, alterations of downstream cascades involving anti-apoptotic or proliferation-promoting signals and their feedback mechanisms, or non-physiological expression of soluble γ_c , which is considered a negative regulator of interleukin signaling. It should be noted that deregulated transcriptional activation of γ_c currently represents the

only common genetic alteration that could be identified in the two SAEs besides the insertional activation of *LMO2*.

5.2. Constitutive activation of *LMO2*

There is little doubt that insertional activation of *LMO2* puts the cells at risk of developing a T-ALL-like disorder. As discussed above, *LMO2* is an established oncogene in childhood T-ALL. However, the long latency of T cell lymphoma development in transgenic *LMO2*-overexpressing mice indicates that further somatic mutations are required for the development of the leukemia-like disorder. It will be important to determine whether all patients in this trial host cells with an insertional *LMO2* activation and if so, whether development of a lymphoproliferative disorder is a default outcome of such an insertional event or cooperation with other oncogenic hits (such as the 6;13 chromosome translocation in patient #4). Importantly, two patients with a follow-up of more than 3 and a half years (patients #1 and #2) showed no sign of SAEs up to the present time.

Assuming that approximately 10 kb are vulnerable for an activating transgene insertion within the *LMO2* locus, such an event could occur in 1/100,000 independent retroviral insertions (or 1 every 100,000 transduced cells having an average of one inserted provirus per cell). Most patients probably received as many as 1,000,000 transduced cells per kg. Following this calculation, 5 to 50 cells per patient may have had a random integration within the *LMO2* locus upon administration. How many of the transduced cells actually engrafted is, however, a matter of speculation. Engrafting efficiency and cell dosage could have played a major role in determining the number of cells with an insertion into the *LMO2* locus actually surviving in each patient. All 15 patients treated in France and U.K. will have their cells analyzed for integration within *LMO2*.

5.3. Age-related, disease-specific and protocol-specific risk factors

The patients that developed the SAEs are those treated at the earliest age (1 and 3 months). Newborns and infants in the first year of life have a very active thymic function. This, together with the selective proliferative stimulus provided by the constitutively active γ_c transgene, could have increased the efficiency of engraftment of genetically modified progenitors, and therefore the likelihood for a cell with an insertional activation of the *LMO2* gene to survive and expand. The rapid reconstitution kinetics of lymphocytes in X-SCID patients receiving gene therapy could be an important age- and disease-specific risk-promoting factor. This hypothesis needs to be addressed in animal models before designing potentially alternative treatment protocols.

Newborns affected by X-SCID appear to have increased numbers of CD34⁺ progenitor cells, likely resulting from the block of differentiation due to lack of a functional γ_c gene. These progenitors have a transcriptionally active *LMO2* gene, like normal hematopoietic progenitors. Therefore, the *LMO2* locus may be at higher risk of retroviral insertion compared to more differentiated cells. Moreover, differentiation-arrested cells may have an increased risk for abnormal growth regulation following γ_c reconstitution, which may diminish a few months after birth. However, a delayed treatment could increase the risk of severe infections in these patients, as well as the risk of failure or reduced benefit of the therapy.

Since insertional mutagenesis is a random event, the risk is expected to decrease if the number of transduced cells is reduced. A better purification of long-term repopulating hematopoietic stem cells could reduce the insertional oncogenesis risk by a factor of up to 100. However, high purification protocols are not common practice in clinical bone marrow transplantation, and should be developed and tested specifically for gene therapy applications.

The retroviral vector used in the X-SCID trial contains wild-type LTRs as a means to control the transgene expression. The available data strongly suggest that the strong enhancer contained in the LTR might be the major cause of the insertional activation of the *LMO2* gene. Future research should address the possibility of disabling the viral LTRs, and replace them with cellular, differentiation-controlled enhancer/promoter elements such as those controlling the γ_c gene itself. However, LTR-defective (self-inactivating) retroviral vectors are known to be more difficult to produce at a titer

compatible with clinical applications, and their use could theoretically introduce additional safety or efficacy problems, such as insufficient gene transfer into the relevant target cells. Potential alternatives, such as self-inactivating lentiviral vectors, are not approved yet for clinical use and have not been shown to be superior for the disease under consideration.

6. Issues of data availability and accessibility in the European Union

The issues of transparency and accessibility to the gene therapy clinical trials data were discussed in details. As planned in the Directive 2001/20/EC on clinical trials, a European database of clinical trials is going to allow regulators to have information circulating through controlled channels in order to have access to clinical and experimental data and all the relevant information about trial outcome and SAEs, in a timely manner. Further reflexion is needed to allow clinical investigators to have access to or to exchange this type of information, in order to be informed if SAEs have occurred or trials have been stopped in any of the EU countries. Further initiative at the European level could be undertaken to facilitate the circulation of the information on these matters/topics.

Conclusions based on current scientific knowledge

The Gene Therapy Expert Group agreed on the following expert opinion:

- Gene therapy of X-SCID is an unprecedented success as a treatment of a fatal genetic disorder, providing a cure for 9 out of 11 treated patients in the French trial alone. The risk of developing a lymphoproliferative disorder as a SAE of the treatment was unpredictable and unexpected. Although high at the moment (20% of the successfully treated patients), this risk, if retaining this frequency, is not higher than the one associated to the only alternative therapy for the eligible patients, i.e., mismatched bone marrow transplantation. The patients that developed the SAE responded to chemotherapy, and maintain the option of undergoing bone marrow transplantation.
- The leukemia-like lymphoproliferative disorders that developed in two patients treated with genetically modified bone marrow cells for X-SCID has likely been initiated by the insertional activation of the proto-oncogene *LMO2* by the gene transfer vector. It is also likely that the growth advantage provided to T cells by the therapeutic γ_c transgene may have played an additional role as a disease-specific risk factor in these SAEs.
- The very young age (1 and 3 months) of the patients and the relatively high dose of genetically modified cells administered at the time of treatment might have increased the risk of an insertional oncogenesis event to occur as well as to undergo positive selection in vivo, and may therefore represent age-related and protocol-related specific risk factors of this trial.
- The investigation carried out so far indicates that the X-SCID disease context may be especially prone to the development of leukemia-like lymphoproliferative diseases initiated by insertional activation of a T-cell specific proto-oncogenes, such as *LMO2*.
- Pre-clinical studies in accurately designed susceptibility models should be attempted to validate the cascade of oncogenic events proposed as the cause of the two SAEs. Such models should provide the experimental framework to test possible improvements in the design of gene transfer vectors, cell purification and transduction protocols, and any other factor that could increase the safety of the gene therapy treatment of this disease.
- From the data available at the moment, it is difficult to predict what implications the high frequency of SAEs observed in the X-SCID trial might have for other gene therapy trials using similar vectors and gene transfer procedures. A longer follow-up of the X-SCID and ADA-deficient SCID trials, and more pre-clinical investigation on animal models, should reasonably provide more safety data in the near future. In the meantime, available clinical and non-clinical data suggest that further clinical trials involving the transfer of genes into hematopoietic stem cells

by retroviral vectors might be considered only for life-threatening conditions, preceded by a careful assessment of the additional risk factors (nature of the transgenes, age of the patient, design of the transfer vector) and the potential clinical benefit for each specific patient and each specific disease.

- Retroviral gene transfer into differentiated hematopoietic cells, such as peripheral blood T lymphocytes, deserve consideration for life-threatening conditions. The safety record of this type of gene transfer is established and is based on a relatively large number of patients in several countries. In the presence of clearly identified potential clinical benefits, non-clinical and clinical data warrant consideration for trials involving this type of procedure.
- The GTEG recognizes the need for more pre-clinical investigation in assessing the risk of gene therapy, including more basic research in the development of safer gene transfer vectors. However, as the history of X-SCID SAEs demonstrates, risk assessment cannot be based entirely on the results of pre-clinical investigation and there appears to be no real substitute for clinical investigation in this field. A correct assessment of the risk/benefit ratio for the patients remains the first and most important scientific and ethical criterion in evaluating the feasibility and acceptability of a gene therapy clinical trial.
- Under the auspices of the CPMP, e.g. in relation with the GTEG, a network involving scientists, clinicians and regulators in the European Union would allow more efficient exchange of relevant information about gene therapy clinical trials within the EU, their outcome and any SAE.

B. Germline alteration by gene therapy: A survey of nonclinical testing studies

The possibility of exposure of gonadal tissue to gene transfer medicinal products raises safety concerns about vertical germline transmission. While germline transmission has not been observed in clinical trial participants to date, findings from recent studies have renewed concern. Detection of vector DNA in semen of clinical gene therapy trial participants led to action by the FDA in the form of temporary clinical hold, which was later lifted after scientific discussions (Marshall 2001, May 10th 2002 meeting report from the FDA Biological Response Modifiers Advisory Committee). Detection of vector sequences in semen will probably become more prevalent in future clinical trials with new technologies allowing higher vector titres and new vector types. With more advanced and effective *in vivo* gene therapy strategies, the risk of inadvertent germline alteration might also increase.

Until recently, the possibility of germline transmission has rarely been tested in animal models; however, several newly published studies investigate the issue.

With the aim of evaluating recent developments in nonclinical testing for germline transmission, a literature survey was conducted. The survey identified 8 recently published non-clinical testing studies on potential inadvertent germline alteration using retroviral, AAV, and adenoviral vectors and plasmid DNA. A compilation of the study results and methods used is shown in the table below.

Also a meeting report from the FDA BRMAC (May 10th 2002 meeting report from the FDA Biological Response Modifiers Advisory Committee) on inadvertent germline alterations was reviewed and relevant parts are discussed in this document.

1. Methods used in the surveyed studies¹:

The survey identified germline transmission studies in mouse, rat, rabbit, dog and sheep.

In all 8 studies, gonads were tested for the presence of vector sequence. 7 out of the 8 studies employed PCR (one of them nested PCR) for the testing of gonads. In addition, RT-PCR, FISH, staining for LacZ expression, immune staining, and southern blot were used.

Sperm cells or oocytes were tested for presence/expression of vector DNA in three studies, using PCR, histochemical, immune fluorescence and blotting techniques.

¹ Details in Annex I

One study investigated mouse seminiferous tubules and epididymal sperm by histochemical staining at different time points after adenoviral vector administration. Another study investigated AAV vector in semen by PCR at different time points after administration, to include one spermatogenesis cycle. Three studies performed *in utero* gene therapy, with, respectively, injection of retroviral vector into foetal sheep, of AAV vector into mouse embryos and of a plasmid vector into rat foetuses. PCR analysis of gonads or semen was conducted for detection of vector DNA from the *in utero* treated animals. Furthermore, breeding experiments were conducted in all *in utero* gene therapy studies to determine if offspring of *in utero* treated animals were transgenic, which would demonstrate that germline transmission had taken place. In these cases, PCR analysis investigated the presence of vector DNA sequences in the offspring.

Of the 8 studies, breeding studies were performed in five of them, one of them being conducted on *in vitro* transduced and fertilised oocytes.

2. Results

Although vector DNA was detected in the gonads in 7 out of the 8 studies, there was no evidence of germline integration in all but one study: germline transmission to offspring of treated rats was reported by a breeding experiment with a study using plasmid DNA. However, the significance of the finding of germline transmission in this case is considered questionable (see discussion).

3. Discussion:

Inadvertent germline transmission is of particular concern when gene transfer vectors, with the potential to integrate into the host chromosome, are administered by systemic routes. The potential for host chromosome integration range from very likely to highly unlikely for the different vector types and must also be accounted for in considering the amount of (pre)-clinical testing needed.

Also, the risk assessment depends on the gender. Currently, there are no non-invasive means to monitor women for germline transmission, and therefore, the risk assessment may be exclusively based on nonclinical animal models. FDA encourages development of appropriate animal models in this area (May 10th 2002 meeting report from the FDA Biological Response Modifiers Advisory Committee). It appears that the probability for vector to get access to oocytes is very slight. Access to the ovarian tissue via the blood stream requires that the vector breach the wall of the blood vessel, traverse the thecal layer, breach the follicle wall, penetrate the layer of follicle cells around the egg, and then enter oocytes which are not yet surrounded by a zona pellucida. Results of intraovarian injection of adenoviral vectors did not indicate this to be possible, as described in the survey table (Gordon 2001). Large amounts of vector were administered directly to the thecal cells, yet cells within the follicle were negative for any signs of vector entry. Vector was also placed under the ovarian bursa as a model for access to the oocyte surface from peritoneal fluid; the findings were negative here also.

Transduction of mature sperm is a theoretical risk with AAV, lentiviral and adenoviral vectors, as they do not have a requirement for cell division in order to transduce cells. Gamma-retroviruses transduce only dividing cells, thus mature sperm cells are unlikely targets for transduction by these viruses; however lentiviruses have the capacity to transduce non-dividing cells. Nevertheless, a gamma-retroviral vector via hematogenous spread theoretically could transduce spermatogonial stem cells, which are rapidly dividing. Spermatogonial stem cells generate differentiating spermatogonia which become spermatocytes. These undergo meiotic recombination, followed by formation of spermatids which evolve into spermatozoa. The earlier the stage that germline transmission takes place in the spermatogenesis process, the greater the risk that the germline alteration is permanent and the greater will be the fraction of transduced sperm cells.

Attempts to infect germline stem cells with viral vectors for transgenesis studies have hitherto been largely unsuccessful, although studies performed with retroviruses to derive transgenic mouse strains showed some success (Soriano et al 1987) and recent studies show that special techniques can be used in order to accomplish this (Nagano et al 2000).

Attempts to create transgenic animals with adenoviral vectors after *in vitro* exposure of mature sperm to the virus or by direct injection into testicular tissues have failed to show significant gene transfer to either spermatozoa or offspring (Hall et al 2000, Peters et al 2001). Safety studies of AAV vectors, including the one used in a recent clinical trial showing vector DNA in semen, have failed to

demonstrate germline transmission in animal models (Arruda et al 2001, Favre et al 2001), as also described in the survey table below.

Considering the physical barriers that a systemically administered vector would need to cross, type A (renewable stem cell) and B (committed to meiosis and spermatogenesis) spermatogonia would be potentially accessible for transduction since these progenitor germ cells are on the blood side of the Sertoli cell barrier (Gordon 1998). Meiotic cells are difficult to access except retrograde through sex ducts.

Since one cycle of spermatogenesis takes approximately 64-74 days in man, the timing of the appearance of transduced progenitor daughter cells in the semen is predictable, which can be taken into account in clinical trial protocols. A similar approach was used in one of the nonclinical studies surveyed in the Table below (Peters et al 2001). This study analysed expression in mice of an adenoviral LacZ reporter gene under a germ cell-specific protamine promoter in seminiferous tubules and epididymal sperm. To differentiate between infection of stem cells and differentiating spermatogenic cells, expression of the reporter cassette was analysed at different timepoints after viral delivery. There was no β -gal expression in either developing spermatids or mature epididymal spermatozoa. Likewise, Arruda et al investigated sperm at different time points based on the duration of spermatogenesis (Arruda et al 2001). The methods used in these studies present a useful tool not only for studying presence/expression but also for identifying the stage of spermatogenesis where germline transmission might have taken place.

Recently, studies investigating the possibility of *in utero* gene therapy have been conducted. *In utero* gene therapy heightens concerns about the risk of germline transmission. However, the timing of vector infection is important; early studies in mice demonstrated that germline transmission was high when pre-implantation embryos were infected with retrovirus and very low (<0.4%) when post-implantation embryos were infected (Jaenisch et al 1981, Soriano et al 1987).

Until compartmentalisation of the primordial germ cells in the gonads, which is completed in humans by the 7th week of gestation, cells are unprotected and mitotically active, allowing viral vector infection. This must be taken into account and *in utero* gene therapy should be conducted after this time-point in order to minimise the risk of germline transmission; additionally it should be taken into consideration that this risk also might apply for other types of gene therapy in fertile women.

In utero gene delivery appears to be inhibited by the presence of amniotic fluid, as shown in a non-human primate model (Bennet et al 2001).

Of the 8 surveyed studies, 3 were *in utero* gene therapy studies, using gamma- retroviral-, AAV- and plasmid- vector injection. Of these three studies, only the one using plasmid transfection showed evidence of germline transmission. However, the significance of the finding of germline transmission in this case was questionable taking into consideration the design of this single study, since it appears that the gonads from the animals giving rise to the transgenic animals did not test positive for the vector sequence.

4. Conclusion from survey and impact on current recommendations for studying vector DNA distribution and germline transmission

In order to evaluate the recent development in testing methods, the methods used for the studies in the above literature survey were compared to those recommended in the European Note for Guidance on gene transfer medicinal products.

The studies of the literature survey used different methodologies for different constructs and were not designed to fulfil regulatory environment. Therefore, no direct regulatory action based on those studies can be made. However, some general trends and conclusions can still be drawn from the survey, providing a basis for further work on recommendations. That is the aim of this report.

Nonclinical studies on vector DNA distribution and germline transmission, as outlined in the European NfG on gene transfer medicinal products:

The nonclinical section of the European NfG on gene transfer medicinal products recommends studying distribution and localisation of the gene transfer product. Specifically, it writes:

“Reassurance regarding the lack of germline alterations may be gained from investigating the presence

and persistence of a NAT² signal for the vector and/or expressed gene product in male and female subjects. A NAT and/or RT-NAT signal in gonadal tissue should be evaluated.”

“Positive findings require further investigation to exclude the presence of the gene transfer product in actual germ-line cells and integration into the genome of germ-line cells. Examination of sperm and ova using NAT techniques may be considered.”

“Such elucidation (...i.e. the possibility of germ-line alteration...) is necessary before commencing any study with the product in human subjects”.

From the current literature survey, it is clear that testing of gonads for the presence/expression of vector sequence does not give sufficient information as to whether the germline has been altered. Although gonads tested positive for the vector sequence in 7 out of 8 studies, further more detailed testing, either of the germ cells or of offspring in breeding studies, gave no indications of germline alteration except in one plasmid gene transfer study.

PCR assays to study vector presence do not provide sufficient information concerning evidence of cell transduction and expression of vector genes; hence, if vector is detected in gonads, more detailed information will be needed.

In male animals, positive semen samples should be fractionated to remove transduced white blood cells from the sperm fraction. This recommendation is in line with current proposals (FDA Biological Response Modifiers Advisory Committee) and a similar approach should be used in the nonclinical testing studies. Testing of fractionated semen should span at least one complete cycle of spermatogenesis.

In female animals, vector injected directly into the ovaries, as published (Gordon 2001), might give an indication of whether it can penetrate the barriers to the oocytes.

A positive signal in the germline cells will require elucidation of whether stem cells are transduced. This can be accomplished in male animals by methods similar to those used by Peters et al (Peters et al 2001).

PCR tests might non-specifically show presence/expression of vector sequence in contaminating non-germline cells. Other expression tests appear to be less sensitive (and reliable) than PCR tests for DNA. More definitive testing such as breeding studies to determine whether offspring of the treated animals are transgenic should therefore be considered. In the literature survey, breeding studies were commonly used to test for germline transmission. Breeding studies provide the most reliable indication of germline transmission, especially for germline alteration in females; however, the sensitivity of breeding studies is very low compared to PCR approaches (a study of several hundred progeny would be a large undertaking, whereas 300,000 sperm cells can be tested in a single PCR reaction).

Preliminary considerations

A decision on when to study vector DNA distribution linked to potential germline transmission should follow risk assessment of the gene transfer medicinal product with respect to vector type, dose, route of administration and medicinal purpose.

There are many different types of vector and genetically modified cells proposed for gene therapy protocols. The relative risk of potential germline transmission for each vector/cell category might be based on whether

1. the vector/cell is replicating or non-replicating, and
2. the vector is preferentially integrating or non-integrating³. For example, cells transduced *ex vivo* with a non-replicating plasmid DNA or non-replicating viral vector would represent the lowest risk category whereas a replicating viral vector with DNA integrating capacity administered intravenously in high dose would represent the highest risk category.

Route of administration is an important consideration. Any parenteral administration of vector/cell could potentially lead to its presence within the gonads, however gonadal persistence would be

² NAT: nucleic acid amplification technique

³ Definition in this context will be further discussed by the GTEG.

determined by dose levels, the route by which the vector/cell enters the body and any specific tropism associated with the vector/cell.

The risk-benefit ratio of the product in relation to the disease/medical condition treated should be determined since this may strongly influence the decision to investigate potential germline integration and transmission of vector DNA.

The Decision tree below gives an elementary breakdown of the perceived product-related considerations for when biodistribution and germline transmission studies might be required.

Following a decision, which should stem from a full risk assessment, to proceed with a biodistribution study, nonclinical model systems, should be developed in accord with recommendations made in the European NfG. In the absence of published 'platform studies', biodistribution studies require the careful selection of animal species in which to model the clinical application and well-validated procedures and tests to enable the distribution of the product within the animal to be reliably and quantitatively determined.

If from the biodistribution study there is no gonadal signal by PCR testing, this might exclude the need for further studies. If however a positive signal is present in gonadal tissues, this will necessitate further steps. A first consideration should be whether the signal persists in the gonads; if the signal strength decreases with time to negligible levels, this might suggest, in the context of the vector properties, only limited further investigations are required. In cases where the signal persists, or where there are known safety concerns regarding the activity of the vector (e.g. integrating) and/or its transgene (e.g. toxic, oncogenic), several approaches, as indicated in the literature survey, should be considered for studying potential vector DNA integration into sperm or ova and germline transmission. The use of other techniques, in addition to PCR, is recommended to confirm positive/negative results. A suggested approach to studying germline tissues and cells is outlined in the decision tree and described in more detail in the flow scheme given below:

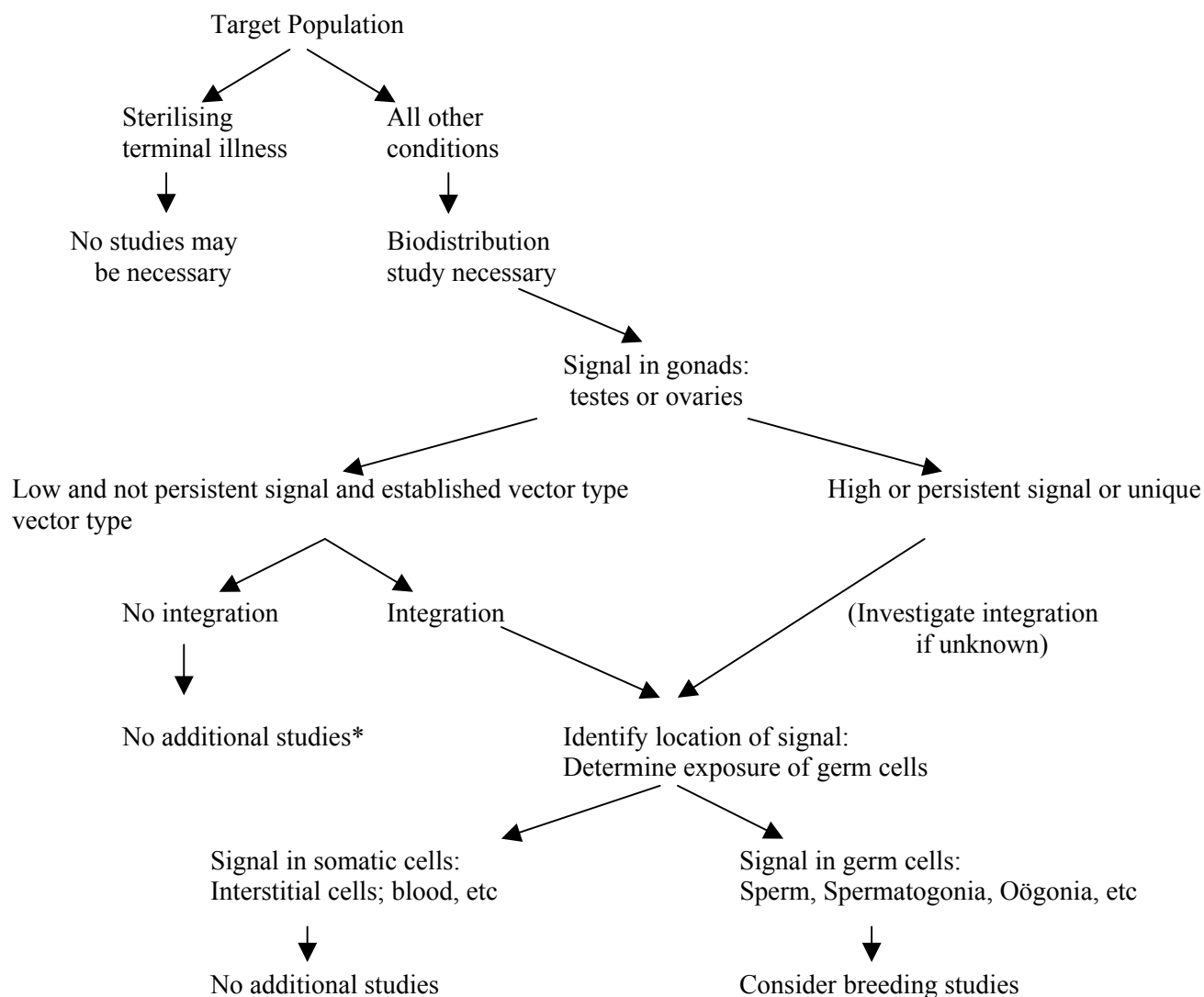
- Testing of gonads for the presence and persistence of vector DNA by PCR and immunological techniques.

If vector DNA is detected in gonads, more detailed information will be needed:

- Testing of germline and non-germline accessory cells (PCR, other?): e.g. testing of semen and ova.
- In male animals, positive semen samples should be fractionated to isolate the sperm fraction.
- In female animals, vector injected directly into the ovaries might show if it can penetrate the barriers to the oocytes.
- When studying the germ cells (from spermatogonia and oögonia), it should be determined if vector uptake is intracellular, and if vector DNA is integrated in the genome.
- If a positive signal in the germline cells is detected: determine if germ cells, ova or sperm are transduced. Test for vector DNA expression (RT-PCR, FISH, etc)
- If a positive signal in the germline cells is detected: carry out breeding studies and look for transgenic offspring with integrated vector DNA

Decision tree for biodistribution and germline transmission studies

Relative Risk	Lowest	Lower	Higher	Highest
Route of Administration	Ex vivo	Parenteral, oral	Systemic	Gonadal; in utero
Vector Type	Non-replicating plasmid; Non-integrating/ non-replicating viral vector	Replicating plasmid; Non-integrating/ replicating viral vector	Integrating/ non-replicating viral vector	Integrating/ replicating viral vector
Dose	Low			High
Target Population	Sterilising terminally ill			Fertile, sexually active



**In case of low and non-persistent signal with a non-integrating vector, no additional studies are requested only if the potential of vector integration into the human genome has been properly investigated and ruled out (see section 5.2.1 of the CPMP Nfg on the quality, preclinical and clinical aspects of gene transfer medicinal products CPMP/BWP/3088/99).*

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ANNEX I

VECTOR	SPECIES	AIM	GONADS	SEMEN	OOCYTES/ SPERM CELLS
Retrovirus ND Tran et al 2000	Sheep	In utero gene therapy. Retroviral Neo ^R and LacZ vector (1x10 ⁷ cfu/ml). Transduction, germline transmission?	PCR	PCR FISH	PCR FISH
AAV	Mouse, rat, rabbit, dog	a) IM 1x10 ¹³ vg/kg. b) Hepatic artery. Biodistribution. Germline transmission?	Positive Sensitive PCR FISH	Positive Sens.PCR Southern blots of PCR	Negative
Arruda et al 2001 AAV	Mouse	In utero gene therapy AAV-EF α -luciferase. Tissue distribution of expression.	Positive PCR	Negative	
Lipshutz et al 2001			Negative		
Adenovirus	Mouse	High dose ornithine transcarbamylase E Δ -E4 Δ vector i.v. Offspring (GD 15): Germ line transmission?	Nested PCR		
Ye et al 1998 Adenovirus	Mouse	AD5-CD/Tkrep intraprostatic. Distribution, persistence, toxicity, germline transmission?	Positive PCR Southern blot Positive		
Paielli et al 2000					

