ARCHIVES OF DISEASE IN CHILDHOOD

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Annotations

Gene therapy for cystic fibrosis

The identification and characterisation of the gene mutated in cystic fibrosis in 1989[1-3] and the development of strategies for the genetic manipulation of mammalian cells have lead to great expectations among patients and physicians for dramatic improvements in the treatment of cystic fibrosis, and in particular have raised hopes that gene therapy for this life threatening disease may be available soon. Last year's successful expression of the normal human CFTR gene sequence (human CFTR cDNA) in the lungs of unaffected rats and mice[4,5] and the creation of transgenic mouse models for cystic fibrosis[6-8] have given further support to the expectation of gene therapy for cystic fibrosis. Now clinicians and scientists involved in treatment and research of cystic fibrosis have taken a further step to make these hopes become reality: in December 1992 three groups obtained approval for phase 1 clinical trials for somatic genetic therapy of cystic fibrosis from the US National Institutes of Health Recombinant DNA Advisory Committee.

Pathogenesis and pathology of cystic fibrosis

To understand the currently proposed experimental strategies for somatic gene therapy of cystic fibrosis, it is useful to recall the molecular pathogenesis of the disease. Cystic fibrosis is autosomal recessive; the gene, which causes disease when mutated codes for an integral membrane protein. Because of its cellular function, it has been called the cystic fibrosis transmembrane conductance regulator (CFTR).[2] The main physiological role of CFTR is that of a cAMP regulated chloride channel.[9] CFTR mutations lead to a disturbance of chloride transport across the luminal surface of the secretory epithelia of the gut, pancreas, lung, biliary ducts, and sperm ducts, and to a compensatory influx of sodium to retain electroneutrality. The accompanying water influx causes, according to one theory, dehydration at the cellular surface and leads to the sticky mucus characteristic of this disease.[10] Other investigators believe that increased pH in the intracellular vesicles as a result of the disturbed chloride transport causes incorrect protein glycosylation, which in turn leads to the cellular dysfunction seen in cystic fibrosis. Atypical glycosylation could lead to an increase in the number of binding sites for *Pseudomonas* spp, and a change of viscosity in the mucus.[11] The secretory sweat gland cells seem to have a CFTR independent mechanism of chloride secretion, whereas an impaired ionic reabsorption in the peripheral part of the ducts is responsible for the pathognomonic salty sweat observed in cystic fibrosis patients.[12]

The sticky mucus in lung, pancreas, and liver causes mechanical obstruction and chronic inflammation of airways and gut lumen, and is the basis for therapy resistant infection, particularly of the lung. Progressive respiratory failure secondary to bronchiectasis is the most common cause of death, with pancreatic insufficiency, hepatic cirrhosis, and diabetes mellitus as other contributing factors. To date treatment of cystic fibrosis is symptomatic, including daily physiotherapy and antibiotics directed against respiratory infections together with pancreatic enzyme supplements and intensive dietary support of the otherwise chronically malnourished patient. More recently amiloride, DNase and α_1-antitrypsin have been on trial as more specific treatments. However, gene therapy is now considered to be a promising long term approach to treatment for cystic fibrosis, particularly with respect to prevention of lung disease.

Approaches to gene therapy of cystic fibrosis

The isolation of the coding sequence of CFTR (CFTR cDNA)[2] was a prerequisite for any strategy directed towards gene therapy. The first attempts towards the correction of the cystic fibrosis phenotype were made on cystic fibrosis cells in culture using retroviral and vaccinia/T7 systems for transfer and expression of CFTR-cDNA.[13,14] Successful complementation of the cystic fibrosis defect in vitro was assessed by restoration of normal cAMP dependent chloride transport to these cells.[13,15] These experiments also established that only one copy of the retroviral CFTR-cDNA construct in cystic fibrosis cells is sufficient to restore normal chloride transport. No harmful effects were observed when human CFTR was expressed in transgenic mice from a lung specific promoter,[16] thereby establishing that overexpression is not a problem. It has also been shown that CFTR expression in normal lung cells is very low[17] and that the presence of less than 10% cells corrected with CFTR-cDNA in a monolayer with uncorrected cystic fibrosis cells is sufficient to restore normal chloride transport in the entire cell monolayer.[18] All these data provide realistic support for the concept of gene therapy for cystic fibrosis.

Somatic compared with germ line gene therapy

Two general approaches to gene therapy can be considered. Somatic gene therapy aims at the correction in a certain type or range of somatic cells expressing the affected phenotype in a particular patient; there is no attempt to correct the defect in egg or sperm cells and therefore correction is not transmit-

ted to any children of an affected individual. In contrast, germ line gene therapy would lead to correction of all cells of an individual, including the germ line. This correction would, therefore, also be propagated to following generations.

At present, clinicians, scientists, and the public agree that there is no medical or ethical justification for manipulating human germ line cells. Our present knowledge of the human genome and genetic disease is not yet sufficient to assume that there are no possibilities of problems in future generations. For the monogenic disease cystic fibrosis, with a risk of one affected fetus in four offspring of a heterozygote couple, preimplantation diagnosis[19] (which would in any case be necessary to decide which fertilised eggs to correct) has made gene therapy at this level redundant. Gene therapy for cystic fibrosis, even if applied in utero for clinical reasons, will involve somatic gene correction.

Ideally somatic gene therapy for cystic fibrosis should permanently replace the affected gene by its healthy counterpart in affected cells. This is, however, not a feasible strategy for somatic gene therapy at present, because of the very low efficiency of such a replacement event (called homologous recombination) when the normal sequence is artificially introduced into a cell. All present strategies are oriented towards complementation of the two mutated alleles by addition of the normal gene sequence.

Virus mediated gene transfer systems

The presently most favoured strategy for gene therapy is to use virus DNA carrier molecules (virus vectors) into which the gene sequence of interest has been incorporated in order to allow its introduction and expression into the relevant cells. Using retroviral vectors, this approach has been successfully pursued for the treatment of adenosine deaminase deficiency by French Anderson and collaborators,[20] and several trials using this vector for different strategies of cancer treatment are under way. Derivatives of adenovirus, vaccinia virus, herpes simplex virus, and adeno-associated virus are the other main viral vectors presently in experimental use for gene transfer and expression in mammalian cells. Each of these systems has its advantages and disadvantages that must be balanced against the potential benefits of gene therapy for cystic fibrosis.

Retroviruses have the principle advantage of a high rate of transfer into mammalian cells and of a possible lifelong cure because of integration into the host genome, provided they infect the relevant stem cells. However, the integration into host DNA occurs at random, which is known to have a mutagenic effect, and also leaves the remote possibility of carcinogenesis by interference with oncogenes or tumour suppressor genes. Another possible, if remote, source of danger would be the recombination of the replication incompetent and therefore harmless virus vector with contaminating helper virus (needed for virus packaging) to give an active tumour virus. These possible risk factors would, however, not rule out the use of retroviruses to treat such a life threatening disease as cystic fibrosis. The fact that retroviruses require cell replication for infection, which does not occur to a marked extent in respiratory epithelial cells, is the most cogent argument limiting their application in vivo for cystic fibrosis.

Adenovirus is presently the most favoured system for prospective gene therapy of cystic fibrosis as it naturally infects lung epithelia. Adenovirus based vectors have been used to introduce human CFTR and α_1-antitrypsin cDNAs into the lung epithelium of cotton rats.[4,21] Expression has been shown to last for up to six weeks, but as the adenovirus does not integrate into the genome of the lung epithelial stem cells it will be lost due to degradation or the regular replacement of epithelial cells. Repeated reinfection will therefore be necessary, which may cause problems with immune reactions against the treatment. There may also be a risk of a replication competent infectious virus generated as a result of recombination with ubiquitous wild type virus, and the virus protein itself is suspected of inducing inflammatory reactions. This could pose particular clinical problems in the context of cystic fibrosis, as reduced resistance to infection and chronic inflammation are general problems in the care of cystic fibrosis patients. Again, these possible risk factors do not exclude the application of such a strategy for gene therapy of as serious a disease as cystic fibrosis, and indeed this approach is the basis for the phase 1 clinical protocols.

Non-viral gene transfer systems

LIPOSOMES

Because of the disadvantages of viral vectors, alternative non-viral systems, such as liposomes, are being considered for gene therapy. Liposomes are membranous lipid vesicles which enclose an aqueous volume. Cationic liposomes form complexes with DNA and can transfer up to 150 kb into cells.[22] They have little negative effect in vitro on cell morphology or growth and are not deleterious in whole animals.[23] Liposome preparations have been approved for human application in cancer treatment[23-25] and have been used to deliver a CFTR expression plasmid to mouse lung cells in vivo. However, it is unclear at present whether the level or the cellular distribution of expression will be clinically effective using this system.[5]

CELL TARGETING

In contrast to adenovirus, liposomes and other non-viral transfer systems have no tropism for lung epithelial cells and the viral mechanisms for cell targeting, entry, and avoidance of degradation of incoming DNA by lysosomes have to be specifically considered. Furthermore, the use of an ex vivo gene correction strategy for the lung, in which (as with bone marrow or liver) cells are taken out, corrected in vitro and then reinfused into the donor, is not likely to succeed. Therefore, specific targeting of the lung epithelial cells and if possible of stem cells in vivo will be necessary. Several strategies are being developed for targeting, including the use of cell tropism of the adenovirus protein coat without using the viral genome as vector system,[26] the use of cell surface receptor ligands like transferrin,[27] or of antibodies against cell surface proteins.[28] An alternative to cell targeting could be the use of lung specific promotors and enhancers, like the surfactant promotor, to direct the cell specific expression of CFTR.[16]

One main approach of our group to tissue specific transfer of large DNA constructs is based on the use of the bacterial proteins internalin and invasin. These proteins specifically bind to the cell surface and allow bacterial entry into cells by receptor mediated endocytosis or phagocytosis. We have cloned the genes coding for internalin and invasin into gene 3 of the filamentous fd phage. Gene 3 codes for the minor coat protein which mediates initial attachment of the fd filament to the end of an *Escherichia coli* pilus. Internalin and invasin are expressed in fusion with gene 3 protein on the surface of the phage and we are now investigating their ability to mediate binding and internalisation of our phage construct to the apical membrane of epithelial cells (unpublished data). The DNA to be delivered will be bound to a protein of this system via polylysine bridges and will be internalised together with this complex. We are also planning to link inactivated adenovirus, which has been shown to be able to break the lysosomal wall after endocytosis,[29] to the complex. This system should enable physiological uptake to occur

without cell damage. The inclusion of appropriate receptor ligands should allow targeting of these complexes to specific tissues, and there should be no limit to the size of DNA construct which can be delivered.

MAMMALIAN ARTIFICIAL CHROMOSOMES

Another, more long term, approach aims at the construction of an artificial mammalian chromosome (MAC) carrying and expressing a normal CFTR gene in a large piece of natural human chromosomal DNA. Such a MAC will carry the DNA elements required for stable replication and segregation, including a centromere, telomeres, and origins of replication, as well as the gene of interest. Ultimately the MAC should be autonomously replicating, and should segregate non-randomly in mammalian cells. It will not integrate into the host genome, thereby excluding any potential dangers connected with virus vectors, and will give permanent expression and physiological regulation.

One of the main scientific problems of this strategy is the present lack of understanding of the structural and functional requirements of a mammalian centromere, the chromosomal element responsible for the correct segregation of chromosomes to daughter cells at cell division. Our present approach to this problem is to introduce putative mammalian centromere elements into yeast artificial chromosomes (YAC), which are then transferred to mammalian cells by fusion with yeast spheroplasts. It has been observed that yeast and YAC DNA can replicate extrachromosomally but does not segregate after transfer to mammalian cells (unpublished data). The inclusion of functional centromere DNA on the YAC should lead to segregation of the extrachromosomal DNA. Novel approaches to targeting and entry will be of particular interest for DNA of the size of a mammalian artificial chromosome, which will exceed the packaging capacity of any virus system.

Animal models and preparation for clinical application

Recently three laboratories have been successful in the creation of transgenic mice with large insertions into exon 10 of the CFTR gene.[6-8] These three knockout mutations are somewhat different from each other and cause different degrees of disease symptoms in the mice, all very similar to the pathology of cystic fibrosis in man, including abnormalities in cAMP stimulated chloride transport. Although the introduced mutations are different from those found in humans, these models will certainly have great impact by allowing new and relevant analysis of proposed pharmacological and genetic approaches to treatment of cystic fibrosis. It can be expected that the models will be improved to correspond exactly to mutations found in the human CFTR gene and will be used for genotype-phenotype correlations, as well as for testing of new therapeutic approaches.

At the beginning of December 1992 three phase 1 clinical trials for gene therapy of cystic fibrosis were approved by the National Institutes of Health Recombinant Advisory Committee in the USA, and one or two groups in Europe are preparing applications. All these trials are to be performed on the basis of fully informed consent of patients with cystic fibrosis. In each case CFTR-cDNA/adenovirus recombinants will be applied to restricted areas of the nose and bronchial epithelia. The molecular and electrophysiological effects of this 'local' in vivo gene complementation will be intensively monitored. The patients will also be investigated for any immunological reactions and any indication of virus spread within the body and to the environment. The main aim of these trials is not to obtain an individual therapeutic effect, but to gain essential information on efficacy, safety, and dose response of this system as listed in the table. These applications have still to gain FDA approval, but undoubtedly the proposed trials will be going ahead during 1993. Whatever their outcome in detail, they will certainly provide important information on the feasibility of gene therapy with the currently available adenovirus vectors and indicate necessary improvements. Although gene therapy may not be the ultimate solution for treatment of cystic fibrosis, these trials will certainly mark the beginning of a new era in the treatment of this disease, as well as providing a model for gene therapy for other single gene disorders.

Main aims of phase 1 gene therapy trials for cystic fibrosis

Overall assessment of safety, efficacy, and dose effect	
● Safety:	Immediate cell damage?
	Virus replication leading to spreading?
	Virus replication causing pathogenic virus?
	Purity and sterility of virus preparation?
	Immunogenicity of preparations?
	Environmental spread?
	Hazards of delivery?
● Efficacy:	Does construct correct defect?
	Which parameters are corrected?
	How long does correction last?
	Is degree of correction likely to be of therapeutic value?
● Dose effect:	Effect of increasing doses of recombinant virus on safety and efficacy

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Haemophilus influenzae type b

The addition of a vaccine for infants against *Haemophilus influenzae* type b is among several significant changes made in recent years to the routine schedule of immunisation in the UK and the Republic of Ireland. In Western countries *H influenzae* type b has been the most common cause of bacterial meningitis and acute epiglottitis in early childhood and also a leading cause of pneumonia, septic arthritis, and cellulitis.

Since 1 October 1992, children <4 years of age in the UK and the Republic of Ireland have been eligible for immunisation against *H influenzae* type b. Two conjugate vaccines are in use: PRP-T (Pasteur-Merieux, a conjugate of the purified polysaccharide, polyribosylribitol phosphate (PRP) linked covalently to tetanus toxoid) and HbOC (Praxis-Lederle, sized oligosaccharides derived from PRP conjugated to a non-toxic variant of diphtheria toxin, CRM_{197}). Both were shown to be highly protective in efficacy studies.[1,2] Furthermore, there is evidence from Finland that implementation of a vaccination programme in the community could go close to eradicating *H influenzae* type b disease.[3] However another *H influenzae* type b vaccine PRP-D (a conjugate of PRP and diphtheria toxoid), which was highly efficacious in Finland, was poorly protective when studied in a different ethnic group (Alaskan Eskimos).[4]

Several important questions remain to be answered.

(1) How well will *H influenzae* type b conjugate vaccines perform in the field in the British Isles?

(2) How can their efficacy be measured?

(3) Is there any increase in the incidence of *H influenzae* type b infection in the period from vaccination until immunity has developed? (In infants <13 months, they are incompletely immunised until 1 week after at least two, and preferably three, doses have been given. In an older child, 2–3 weeks must elapse before protection develops after a single dose.)

(4) Does invasive infection that occurs despite 'incomplete' vaccination result in immunity?

(5) Is a booster dose of vaccine required in the second year of life?

A special opportunity now exists to address these questions through a British Paediatric Surveillance Unit (BPSU) study of invasive *H influenzae* type b disease occurring after vaccination against *H influenzae* type b. Contrary to the practice in the USA and mainland Europe, the UK and the Republic of Ireland offer only primary immunisation against *H influenzae* type b with no booster dose in the second year of life. This is despite the fact that primary immunisation is now completed at a much younger age (4 months in the UK and 6 months in the Republic of Ireland), another recent and significant change to the routine immunisation schedule. Conjugate vaccines induce T cell dependent memory and thus there is the potential for a booster response should the *H influenzae* type b organism or cross reactive antigen be encountered subsequently. There are at present no strong epidemiological data to support the need for a booster dose. It is clearly vital that cases of invasive *H influenzae* type b infection in children who have been appropriately immunised are notified. Complete reporting of such 'true' vaccine failures will enable a meaningful audit of vaccine efficacy and determine if waning immunity (after primary immunisation has been completed) can result in children becoming susceptible to invasive *H influenzae* type b disease.

Auditing of interventions that purport to promote health is becoming an integral part of modern medical practice. Post-marketing surveillance through the BPSU study in the British Isles offers several distinct advantages over that being performed elsewhere in the world (for example, in the USA where various *H influenzae* type b conjugate vaccines have been offered since 1987 in a piecemeal and evolving way).

These advantages include the following:

(1) Definition of the target population is a straightforward task because of the defined date (1 October 1992) for beginning vaccination and the clear guidelines given as to who is eligible (children <4 years).

(2) The vaccination status of any particular child in the UK can usually be easily ascertained by reference to district computer records with only occasional recourse required to parent held, general practitioner, or health visitor records.

(3) The childhood immunisation programme includes a standardised approach across the UK that is centrally organised but also involves the provision of excellent peripheral support through the work of immunisation coordinators (health professionals specifically designated to advise on and promote immunisation) in each health district.

(4) A guide for UK doctors, *Immunisation against Infectious Disease*, is widely distributed and regularly updated.[5]

(5) There is excellent professional and consumer confidence in the childhood immunisation programme and very high rates of immunisation uptake (90% and greater) are now being achieved in most districts of the UK. The rates are lower in the Republic of Ireland.

(6) In the UK and the Republic of Ireland, infants <13 months of age are only being offered one *H influenzae* type b conjugate vaccine (PRP-T in the UK and HbOC in the Republic of Ireland) so any impact on disease in this age group by a particular vaccine is easier to analyse. Older UK children (between 1 and 4 years) are offered one injection with either one of the two vaccines PRP-T and HbOC whereas only HbOC is offered in the Republic of Ireland.

(7) The sheer scale of the childhood immunisation programme in the British Isles (more than 3 million children aged less than 4 years of age) means that important questions