

# Research Approaches towards a Cure for Duchenne Muscular Dystrophy

A report on the state of international research for the development of  
a causal therapy of Duchenne muscular dystrophy

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for the German Duchenne Parent Project

Aktion Benni & Co

and dedicated to all Duchenne boys and their families all over the world  
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This report has been written for families who have one or more boys with Duchenne muscular dystrophy. It explains some basic scientific facts and shows the now numerous approaches with which research is trying to find a *scientifically justified* and thus effective therapy of Duchenne muscular dystrophy. Because scientists in more than one hundred laboratories in many countries of the world are working to find a cure for this disease, only the most important of their results are described here in greater detail. Some further

results are reported in a strongly abbreviated fashion.

The first edition of this report in 2001 was based on an international workshop on Duchenne therapy research in May 2000 at the *National Institutes of Health* in Bethesda near Washington.

**This third edition of the report was written in July and August 2003** mostly with information from the literature and by correspondence with many of the researchers.

## Introduction

**Some basic scientific facts are explained, what genes are, how they work, why dystrophin is important, how mutations cause Duchenne muscular dystrophy, and how the disease is inherited.**

**Genes and their function:** Genes are functional units of the genetic material in the chromosomes of each cell. This material is *desoxyribonucleic acid*, DNA. Its structure looks like an intertwined ladder, the *double helix*. It was detected by *James Watson* and *Francis Crick* in 1953, 50 years ago. The two backbones or strands of the ladder are long chains of phosphoric acid and desoxyribose, a kind of sugar. The rungs consist of four different chemical substances, the *bases* or *genetic letters*: *adenine*, *guanine*, *thymine*, and *cytosine*, abbreviated A, G, T, and C, two of which always face each other in one rung of the helix. For spatial reasons, the rungs can only contain the pairs A-T or G-C. If the sequence of these *bases* on one strand is e.g.

---AGGCTTAATCGT---

the sequence on the opposite strand must be

---TCCGAATTAGCA---

i.e., the sequences are *complementary* to each

other.

Each of the about 100 trillion ( $100 \times 10^{12}$ ) body cells in a human being contains in its nucleus 46 chromosomes with a total of more than 6 billion genetic letters, grouped in about 25,000 to 35,000 genes. Almost all details of the sequence of these letters are now known. It is the *genetic information*, which is passed on from generation to generation with very little changes or mutations. These mutations, which were necessary for the evolution of all living beings, can also have negative consequences as, e.g., hereditary diseases.

Most of the genes carry the information for the construction of one or more *proteins*, which consist of amino acids. The sequence of the amino acids, of which there are 20 different kinds, is important for the function of the proteins such as *enzymes*, the catalysts for biochemical reactions in the body, as regulators for

other genes, or as structural material.

In the cell nucleus, where the chromosomes reside, the genetic information of the genes is copied or *transcribed* to another genetic substance of a similar structure, the *pre-messenger ribonucleic acid*, pre-mRNA. The genes of multicellular organisms consist of active sections, *exons*, and inactive ones, *introns*. After the transcription, the introns, which are often much longer than the exons, are removed from the pre-mRNA, and the transcribed exons *spliced* together to the *messenger RNA*, mRNA, which is then exported to the ribosomes, the protein synthesizing structures in the cytoplasm of the cell. In the ribosomes, catalytic acting RNAs, *ribozymes*, use the genetic information of the mRNA to construct specific proteins out of amino acids which are delivered to the ribosomes by another kind of RNA, the transfer RNAs or tRNAs.

The RNAs use the base U, uracil, instead of the very similar base T of the DNA. In the mRNA, three consecutive genetic letters always signify one of the 20 different amino acids according to a genetic dictionary, the *genetic code*, which is the same for all life on earth. Thus, the genetic script uses only four letters, and its words, the *codons*, are always three letters long, *triplets*. There are no spaces between the words, and three different *stop codons* exist, UAA, UAG, and UGA, where the protein synthesis is terminated.

**Dystrophin gene:** Duchenne muscular dystrophy is one of the most frequent hereditary diseases. About one in 3,500 boys is born with this disease, which is caused by a *mutation* or damage of the *dystrophin gene* with the consequence that the protein dystrophin is no longer present or exists only in traces in their muscle cells.

The dystrophin gene was *identified* in 1986 on the X chromosome (*Kunkel*, Boston) and its structure elucidated shortly afterwards (*Hoffman*, Washington). With 2.6 million base pairs, it is the longest gene of man. Only 0,5 % of the base pairs, 13,973, belong to the 79 exons of the gene, which contain the active coding sequence, the information for the synthesis of the different forms of the protein *dystrophin*. The transcription of the genetic information of the dystrophin gene into mRNA is under the control of five *promoters*, DNA regions governing the splicing process so that a number of dystrophins of different

length are produced. The main product is the full-length dystrophin, a very long protein consisting of 3,685 amino acids.

Dystrophin is part of the *costamers*, which connect the Z discs of the *sarcomers*, the contractile structures, with the *sarcolemm*, the cell membrane. It is thus important for the mechanical stability of the muscle cells during muscle contraction.

**Dystrophin network:** Dystrophin belongs to a network of many different proteins of which more than 50 are known. Among them are the dystro- and sarcoglycans, the synthrophins and integrins, dystrobrevin, nitric oxide synthase, and other components such as dysferlin, sarcospan, laminin, caveolin, telethonin, myotolin, agrin, neurexin, desmuslin, syncoilin, fukutin, aquaporin, spectrin, collagen, calpain and others. In the future, more components are expected to be identified (*Campbell*, Iowa City).

When dystrophin is missing, the balance between the different parts of this dystrophin complex is disturbed. Especially the dystroglycans, the sarcoglycans and sarcospan are reduced or disappear completely. Every one of the proteins of the complex has its own gene which also can be disturbed by mutations. This leads to the presently known 13 different limb girdle and 5 congenital muscular dystrophies, as well as to at least 8 other neuromuscular diseases.

**How big are molecules, DNA and dystrophin?** A lay person seldom has a correct idea of the sizes of molecular structures with which scientists are working.

The following mental experiment demonstrates the smallness of a simple molecule. Pour on quarter of a liter of wine at the straits of Gibraltar into the Mediterranean. Then mix the Mediterranean well and, at the other end in Alexandria, take out one quarter liter of water with the same wine glass. How many alcohol molecules will you find in the wine glass? Twenty two million!

The DNA double helix has a diameter of two nanometers, millionths of a millimeter. If one enlarges the helix to a diameter of one centimeter, how tall would a person 1,80 meters tall be if one would enlarge him/her by the same factor? Nine thousand kilometers, this is about the distance from Europe to Florida.

Each cell nucleus of the about 100 trillion ( $100 \times 10^{12}$ ) cells of an adult human being con-

tains the complete genetic material with 6 billion base pairs. The entire DNA in all chromosomes of each single cell nucleus is two meters long!

Every dystrophin protein is 125 nanometers long, thus 80,000 of them laid down in a straight line would cover just one centimeter.

There are 114 billion dystrophin molecules and the same number of dystrophin protein complexes in one gram of muscle.

**Mutation and origin of the disease:** Duchenne muscular dystrophy is caused by three different kinds of mutations of the dystrophin gene: *deletions*, if one or more of the exons of the gene are missing, *duplications*, if parts are duplicated, and *point mutations*, if single bases are exchanged, eliminated or added. As the reading mechanism of the information in the ribosomes always reads code words of three letters one after the other without spacings, a mutation does not upset the *reading frame* if the number of letters missing or added can be divided by three without a remainder. In this case, the dystrophin made is longer or shorter. If this change only involves non-essential structures of the dystrophin as e.g. the central part, it can still be partly functional. Then, the benign form of dystrophy, *Becker muscular dystrophy*, develops.

If the mutation shifts the reading frame by one or two bases, then a whole string of incorrect amino acids is incorporated into the protein starting at the mutation site until finally a new and premature stop codon is reached which terminates the synthesis. The incomplete dystrophin cannot fulfil its normal function, it is degraded and *Duchenne muscular dystrophy* develops.

Without dystrophin, the muscle cells degenerate. They are continuously regenerated, but the repair mechanism eventually fails. The destroyed muscle fibers are replaced by fat and connective tissue leading to fibrosis and, at the age of two to three years, to the first visible symptoms of the disease.

Where the motor nerves contact the muscle membrane, another protein with a structure similar to dystrophin, *utrophin*, is located and contributes to some extent to the stability of the muscle membrane (Davies, Oxford). Without utrophin, the disease would progress much faster.

**Genetics of Duchenne muscular dystrophy:** In addition to the 44 normal chromosomes, the autosomes, boys have two different sex chromo-

somes in the nucleus of every one of their body cells, one Y chromosome and one single X chromosome. If the dystrophin gene on their X chromosome is damaged by a mutation, it cannot be compensated for by an intact gene on a second X chromosome, as it is possible for mutations on the autosomes, which are always present in pairs. Therefore, Duchenne and Becker dystrophy affect *only boys*.

Women, however, have two X chromosomes in their body cells. When they carry a mutated dystrophin gene on one of their X chromosomes, they can transmit the disease, they are *genetic carriers*. As one of the X chromosomes is inactivated in a random fashion, about only half of their muscle cell nuclei have an intact dystrophin gene. This is sufficient to cause either none or only weak clinical symptoms of the disease.

About two thirds of Duchenne boys inherit the disease because their mother is a *genetic carrier*. At the *meiosis*, the cell division leading to the egg cells, each egg cell receives only one X chromosome. The probability that it is the X chromosome with the gene mutation is 50 %. Therefore on average 50 % of her sons will have Duchenne muscular dystrophy and on average 50 % of her daughters will also be carriers. These risks remain the same for *all* children in a family, it is not smaller if the family already has a son with Duchenne muscular dystrophy.

If the mother is a carrier, the mutation arose either in the germ cells of her parents or in an earlier generation. As all body cells of a genetic carrier have the mutated gene, her carriership can be detected by a gene analysis in the leukocytes, the white blood cells, which contain chromosomes.

About one fourth of Duchenne boys have a *new mutation*. In these cases, the mutation took place *spontaneously* in the particular egg cell of the mother that then became the patient. As only one egg cell is affected, all other children of these women do *not* face a greater risk than the general risk for the disease.

About one tenth of Duchenne boys have a mother with a *germ cell mosaic*, because the new mutation arose early in the germ cell formation and the mutated cell developed into a group of egg cells each carrying the mutation. As more than one egg cell is affected, another son can inherit the disease too, or a daughter can be a genetic carrier.

As present genetic methods cannot detect a germ cell mosaic, a prenatal diagnosis should be offered during a second pregnancy to all women who already have a boy with Duchenne muscular dystrophy, not only to those who are proven genetic carriers (Müller-Reible, Würzburg).

**Clinical course of Duchenne muscular dystrophy:** The first clinical signs appear at about two to three years of age causing difficulties in walking and especially in climbing stairs. Without early detection, even today the disease is generally diagnosed at about 3 to 5 years. Because

of increasing contractures at the foot, knee, and hip joints, the patients lose their walking ability at 10 to 12 years. Increasing spine deformities, scoliosis, and restrictions of movement make them soon completely dependent on intensive care. The involvement of the respiratory and heart functions lead to death by cardiac and circulatory insufficiency at an early adult age. Early orthopedic operations to avoid contractures and spine deformities as well as respiratory aids and other measures can improve the quality of life and significantly prolong life expectancy.

## Gene diagnostics

**Genetic methods for the diagnosis of Duchenne and Becker muscular dystrophy in a boy, during a pregnancy at risk, and for the determination of carriership in a woman. Early detection in infancy.**

**Diagnosis of Duchenne muscular dystrophy in a boy:** For a gene analysis, a blood sample with leukocytes is needed. These white blood cells, but not the red ones, have cell nuclei which contain the hereditary material DNA. The white cells are isolated and the DNA is obtained from them.

As about 60 % of Duchenne patients have deletions of one or more exons in the their dystrophin gene, one looks first for these deletions which can be everywhere in the gene but which are more numerous in some regions than in others. Generally, in a first step, 19 selected exons of the 79 in the dystrophin gene are multiplied, *amplified*. Normally, one does not amplify all these 19 exons simultaneously, but by *multiplex reactions* in groups of 4 to 6 exons.

This already allows the detection of 98 % of all deletions. In two thirds of these cases, one can already deduce whether the reading frame is disturbed or not and thus predict whether the patient has a Duchenne or a Becker dystrophy. For the remaining third, more exons must be amplified.

The amplification is performed by the *polymerase chain reaction*, PCR, which needs *primers* for each exon. The primers are short synthetic DNA sequences which attach themselves to the beginning and the end of an exon sequence. The small gene fragments obtained in this way are separated by electrophoresis where they migrate different distances in a gel layer and then can be made visible as bands like unevenly spaced rungs of a ladder. Each band represents one of

the 79 exons. A deletion is detected when one or more of these bands are missing.

If no deletion is found, a point mutation is probable. To characterize it unequivocally, the base sequences of most of the exons would have to be determined. Such sequence determinations, however, are very demanding, and they are not yet routinely offered. In these cases, a final diagnosis can only be made by investigation of muscle tissue obtained by a biopsy. There, not the dystrophin gene is analyzed, but the dystrophin protein in a *western blot*.

In this method, after electrophoretic separation, the protein pattern is transferred to another carrier material by blotting, where it is made visible with antibodies. Or fluorescent antibodies are attached to the dystrophin, which can then be detected under the microscope as a bright line around the healthy muscle cells. In Duchenne dystrophy, it is not seen there or only in traces, and in Becker dystrophy, these lines are often frayed, interrupted and mostly much weaker than normal.

If a gene analysis gives an unequivocal result, a muscle biopsy is often no longer necessary. Especially, small children could thus avoid this surgical procedure. However, if a muscle biopsy is necessary, then it can also be performed as a *needle biopsy* under local anesthesia.

**Prenatal diagnosis:** For a prenatal diagnosis of Duchenne dystrophy, there generally must be a definite reason mostly because there already is or was a patient in the family. For a diagnosis during early pregnancy, tissue of the unborn

child must be obtained either by a *chorion villi biopsy* in the 10<sup>th</sup> to 12<sup>th</sup> pregnancy week or by an *amniocentesis* in the 13<sup>th</sup> to 16<sup>th</sup> week.

For a chorion villi biopsy, a small piece of the future placenta is aspirated through a fine canula. If it is certain that cells of the child have been obtained, the tissue can be used directly for the analysis. The risk that this intervention terminates the pregnancy involuntarily is about 3 to 5 %.

The advantage of an amniocentesis is the lower abortion risk of below 1 %. The disadvantage is that the few cells of the child in the amniotic fluid must first be multiplied in the laboratory, and this can last up to 3 weeks, therefore, the analysis cannot begin before the 15<sup>th</sup> to 18<sup>th</sup> week.

If sufficient cells of the child have been obtained, the chromosomes are investigated first. This allows to determine the sex of the child. If it is a girl, then, in most cases, no further tests are performed, because the consequences of a Duchenne carriership should be discussed with the girl when she is old enough to understand them and to make her own decisions.

If the unborn child is a boy, then a gene analysis if performed in most cases with the same techniques as described for the diagnosis after birth.

**Diagnosis of carriership:** If the mutation of a Duchenne boy in the family is precisely known, one can specifically look for the same mutation in the mother and in her female relatives. This is technically more difficult than in Duchenne boys because only one of the two X chromosomes of a woman could carry the mutation. If she is a carrier, the intensity of the bands after electrophoresis are only reduced to half of normal if one or more exons are deleted. However, the amplification of the exons is not easy to control. And it is often difficult to detect reliably the differences of the band intensities.

Therefore, one often uses *polymorphic markers* for the analysis. These are DNA sequences in the introns between the exons which are almost always differently arranged on an individual chromosome, and they also have different lengths so that the two chromosomes can be distinguished. These marker sequences have nothing to do with the disease, they are characteristic for each person, and they can also be amplified by the PCR method and thus be identified un-

equivocally after electrophoresis.

If a boy has a deletion in the dystrophin gene, not only are one or more exons missing but also the markers in the introns between them. In a woman, one can check whether these markers are also missing on one of her X chromosomes. If the markers are missing which her son also does not have, then it is highly probable that she has the same deletion as he on one of her X chromosomes. She is thus a genetic carrier. If she is not a carrier, she should have additional markers in this region.

The DNA markers can also be used if the patient in the family does not have a deletion. Then one does not know what the mutation looks like. But based on the arrangement of the markers, one can say on which of the two X chromosomes of the mother the mutated gene is located which her sick son has inherited from her. And every woman in the family who has the same X chromosome has a high risk being a carrier.

This indirect method has been used for about 20 years, one now knows, however, many more markers. Therefore, one can almost always distinguish the two X chromosomes of a woman from each other, and it hardly ever happens any more that a family is *not informative*.

If, however, all these methods are not successful, then, in some cases, one can mark certain gene regions on the X chromosome with fluorescent gene probes and then check under the microscope whether light points are present or missing signifying normal or deleted gene regions. But this *FISH* method – fluorescence in situ hybridization – can only be used if certain specific deletions have happened in the family.

**Sequence analysis of the dystrophin gene as routine test:** With the new analytic technique *SCAIP* (single condition amplification/internal primer) it will in the future be possible to analyze reliably within three days point mutations, small deletions, as well as all other mutations in a routine fashion. In this method, all exons of the dystrophin gene, all intron-exon border regions with the splice signals, and all promoters are completely sequenced. All these gene regions, together about 110,000 base pairs long, are amplified with their special primers in one single PCR reaction and then checked in a micro electrophoresis. This allows the determination of all exon and promoter deletions, also those which are not found with the other methods. The base

sequence of each gene fragment is then determined automatically with individual sequencing primers to detect the point mutations.

As this method also detects all mutations of Duchenne carriers, it will lead to an improvement of genetic counselling. Data files are now compiled which will allow the prediction of the clinical course of a Duchenne or Becker muscular dystrophy based on a defined deletion, duplication or point mutation. For this purpose, many patients will have to be investigated clinically and genetically (*Flanigan*, Salt Lake City).

**Early detection at infant age** (without gene technique): In Germany, 4 weeks to 1 year old infant boys with Duchenne and Becker muscular dystrophy, which do not yet have clinical symptoms, are detected in a voluntary *CK screening program* in which the enzyme creatine kinase, CK, is determined in a dry blood spot. From

1974 until July 2003, more than 500,000 infant boys have been tested. Among them, 178 boys with strongly elevated CK activities were found, for 136 of them, a Duchenne muscular dystrophy (1:3,700) was diagnosed or made probable, and 28 had a Becker muscular dystrophy (1:17,800).

The early detection allows the parents *to make in time the decisions which are the correct ones for them*, so that no other child with the same disease is born in the same family or in maternally related families. In addition, the *diagnostic odyssey* can be avoided which still often takes many years until an expert for muscle diseases is found. Such programs will be necessary in the future, because the progress in therapeutic research will lead to studies with very young patients who have no clinical symptoms and whose muscles are still largely intact (*Scheuerbrandt*, Breitnau/Freiburg).

### **Therapeutic possibilities:**

**Research strategies for a causal therapy.**

**There is still no therapy for Duchenne muscular dystrophy.**

To cure Duchenne muscular dystrophy, the consequences of the mutation of the dystrophin gene, the muscle degeneration, have to be stopped or at least alleviated. Research tries to do this either by *gene therapy* or by *drug therapy*. Gene therapy means that either all exons of the intact dystrophin gene, its *cDNA*, or parts of it, are introduced into each muscle cell, or that the damaged gene is repaired by genetic techniques. A drug therapy would mean that a new or already known drug is given to block or to slow down the muscle degeneration without influencing the gene itself. On both approaches, important progress has been made during the last few years.

**There is no therapy yet:** In spite of this progress, neither a gene nor a drug therapy has been

developed so far which would cure the disease of Duchenne boys. Only three closely related drugs, *prednisone*, *prednisolone*, and *deflazacort*, have been found which, during a limited time, can slow down the degeneration of the muscles.

Because of the severity of the disease, the patients, their families, their doctors, and the public in general are looking desperately for even the smallest positive research result. And the media, mainly newspapers and television, even the responsible among them, tend to exaggerate small advances as important breakthroughs. But also the scientists themselves are often over-optimistic when they declare the results of their experiments with animals as therapeutic victories, thus raising false hopes. *Not a single Duchenne boy has ever been cured.*

### **Transfer of a new dystrophin gene**

**The exons of the entire or the shortened dystrophin gene can be transferred into muscle cells by viruses, plasmids, or myoblasts. Immune reactions must be avoided.**

It is reasonable to believe that the transfer, the transportation, of sufficient quantities of the intact dystrophin gene into the nuclei of dystrophic muscle cells would cure the disease if the genetic information of the new genes is used by the protein synthesizing ribosomes of the cell to pro-

duce sufficiently large quantities of functional dystrophin which then migrates to its normal place under the cell membrane where it is correctly incorporated into the intricate dystrophin-glycoprotein complex of the costameres.

**The dystrophic mouse:** Most of the experi-

ments with this aim have been performed with one kind of laboratory animal, the dystrophic *mdx mouse*, which has a point mutation at base pair 3,185 in exon 23 of its dystrophin gene. This mutation has changed a CAA codon, which signifies the amino acid glutamine, to a TAA codon, which is a stop sign, so that the synthesis of dystrophin is interrupted prematurely. Thus, the mouse has no functional dystrophin in its muscles. However, these mice do not lose their muscles because they do not develop *fibrosis*, a proliferation of connective tissue, like Duchenne boys do, so that the degeneration caused by the disease does not overtake the regeneration.

Although gene transfer can be studied with them, one should keep in mind that any results obtained with these mice cannot be regarded as immediately applicable to children as long as they are not confirmed by clinical studies with Duchenne patients. *A child is not a big mouse!*

**The dystrophic dog:** Some experiments are performed with dystrophic dogs, the *golden retriever muscular dystrophy* (GRMD) dogs which, in contrast to the mice, have a muscular dystrophy similar to the dystrophy of Duchenne boys. They are really handicapped and hard to raise and to manage. Their dystrophin gene has a point mutation in the splice receptor site of intron 6 which leads to the deletion of exon 7 in the mRNA, to a frame shift, and a premature stop codon.

**Gene transfer with adeno viruses:** In order to transfer a gene, a transporter, a *gene vector*, is needed. One way to transfer genetic material into living cells is to pack it into viruses, which consist of a string of their own genes enveloped in a protein shell. They attach themselves to special receptor proteins on the surface of a cell, inject their genes through the cell membrane and then use the synthesizing apparatus of the infected cell to reproduce themselves.

Mainly two kinds of viruses are used as gene vectors in Duchenne research, the *adeno virus* and the ten times smaller *adeno-associated virus*. The vectors derived from them cannot be further multiplied inside the target cell because almost all of their own genes have been eliminated. The most advantageous vector seems to be the *guttled*, practically empty adenovirus, which does not contain any genes of its own, and thus has room for up to 36,000 foreign genetic letters, sufficient for the entire cDNA, i.e., all exons with the in-

formation for the complete dystrophin protein and for additional control sequences. With these gutted viruses new dystrophin was produced in up to 30 % of the muscle fibers of a dystrophic mouse followed by an improvement of muscle function in young as well as in older mice (*Lochmüller*, Munich; *Chamberlain*, Seattle).

**Two genes in one virus:** In new experiments *two* mouse dystrophin cDNAs were packed into each gutted virus, that means, two times all 79 exons without the introns, and in addition two more gene sequences, the very strong enhancer of the cytomegalovirus and the beta-actin promoter. These gene vectors were injected into the tibialis anterior muscle of newborn *mdx* mice and into 4-6 weeks old juvenile mice. After 30 days, 42 % of the cells in the treated muscle of the newborn mice had new dystrophin which was still detectable after half a year. In the juvenile mice, 24 % of the muscle cells had new dystrophin after 30 days, which, however, was reduced to half the amount after 6 months. Only the juvenile mice showed small immune problems. At present, these new vector constructions are the most effective ones for the treatment of Duchenne muscular dystrophy by gene therapy (*Karpati*, Montreal).

**Gene transfer with adeno-associated viruses:** These small viruses can only transport genetic material that is not longer than about 5,000 base pairs, about one third of the entire dystrophin cDNA. Their advantage is that they transfer the gene more effectively than the normal adenoviruses. The disadvantage is that the dystrophin cDNA to be transferred has to be shortened considerably to fit into this small vector. Patients with Becker muscular dystrophy, that progresses much slower than Duchenne dystrophy, mostly have such shortened dystrophin in their muscles. Therefore, a transfer of one of these *Becker mini genes* would not completely cure Duchenne muscular dystrophy but only transform it into the benign Becker form.

In order to determine which of the four regions of the normal dystrophin protein – the two end regions, the cystein-rich, or the central rod regions – are important and which are not, extensive experiments were performed with dystrophic mice which were genetically modified so that they had one of nine different shortened dystrophins in their muscles.

It had already been known that some of the

rodlike central region can be removed without loss of function. The detailed analysis of the three-dimensional structure revealed now which particular parts of the rod structure could be deleted without significantly losing the muscle protecting function. One of the shortened dystrophins, which was about half as long as the normal one, had practically the same properties as the unaltered protein. Delivery of the genes of this and some of the other shortened dystrophins with adeno associated viruses into the muscles of the mdx mice prevented and partially reversed the dystrophic symptoms. These results demonstrated that specific modifications of the dystrophin gene can generate novel proteins that are significantly smaller but more functional than the naturally shortened dystrophins of patients with Becker muscular dystrophy (*Chamberlain, Seattle*).

If about 30 % of the normal amount of unchanged dystrophin re-appears, measurements on the diaphragm of the mice indicated that the muscle function also improved. The transfer of the mini gene also led to an improvement of muscle function. In addition, the gene transfer showed better results in younger animals, because there were fewer problems of immune rejection, and, after a single injection, the newly synthesized dystrophin remained in the muscles longer than in adult animals. After a single treatment of newborn mice, newly formed dystrophin could still be found after one year.

For a future application in humans, this means that the patients would have to be treated as soon as possible after birth when their muscles are still largely intact (*Clemens, Pittsburgh*).

**Gene transfer via the blood stream:** In the experiments so far described, solutions of the viruses that contain the dystrophin gene were injected directly into the muscles. In order to reach all muscles, also those of the heart and the lungs, experiments are being performed to develop a *systemic treatment*, i.e. the injection of the viruses into the blood stream. To ensure that the new dystrophin is only synthesized in muscle cells, another genetic sequence had to be added to the dystrophin gene in the virus, the *CK promoter*, which normally activates the gene of the muscle protein creatine kinase. This promoter would assure that the new dystrophin gene is activated only in muscle cells and not elsewhere.

To investigate whether such strategies would

function in a living animal, *transgenic* mdx mice were created which have an additional gene construction inserted in their chromosomes. This consisted of an often used Becker gene which, with 6,300 base pairs, is only half as long as the normal gene, and which was preceded by a CK promoter of 1,354 base pairs. Shortened dystrophin was then present during the entire life time of the mice of up to 24 months in sufficient quantities to improve all measurable clinical symptoms.

The new protein appeared only in the muscles of the transgenic mice, significantly more in the fast muscles than in the slow muscles. The fast muscles, which can work immediately but not consistently, obtain their energy through glycolysis, through the fast degradation of glucose without the use of oxygen. The energy for the slow and consistently working muscles is provided by the enzymes of the respiratory chain, the slow "burning" of organic substances with oxygen. As the fast muscles are destroyed first by the muscular dystrophy, the predominant appearance of new dystrophin in these fast muscles is able to ameliorate just this early consequence of the disease. Experiments have also shown that as few as 20 to 30 % of the normal quantity of dystrophin have a significant therapeutic effect.

For these experiments with transgenic mice, the Becker dystrophin gene together with the CK promoter was introduced into the germ cells by artificial insemination and genetic manipulation, so that it was inherited to the progeny of the mice. This is a technique which obviously cannot be performed with humans. To cure the sick children, one would have to transport the gene with its promoter by gene transfer with a vector soon after birth, if possible, into all muscles (*Lochmüller, Munich*).

**Amplification of targeted gene transfer:** The viruses attach themselves to specific structures on the cell membrane, to the coxsackie and adenovirus *receptors*, from where they penetrate the muscle cell through the membrane. These receptors, however, are more numerous on developing and regenerating muscle cells. They are downregulated, less numerous, on mature muscle cells which no longer divide. Therefore, gene transfer with adenovirus vectors is more efficient in dividing muscle cells.

To overcome this handicap, *transgenic* mice were produced which expressed these receptors

in large amounts on the surface of mature muscle fibers. This led to an up to 10 times more efficient transfer of the dystrophin gene (*Holland, Montreal; Lochmüller, Munich*).

As a multiplication of the of the virus receptors on the muscle cells of a patient could only be achieved by additional gene technical methods with all their risks, helper proteins were produced. In this case, they were monoclonal antibodies which consisted of only one kind of molecule that could bind with one end highly specifically on other, more numerous receptors. With their other end, these immune proteins could bind to modified coat proteins of the adeno viruses. To achieve this binding, additional genetic information for a chain of 33 amino acids from a bacterium was introduced into the adeno viruses by genetic manipulation.

These adeno viruses could now attach themselves via the antibody bridge to integrin and nerve receptors (NCAM) which were much more numerous than the adeno receptors. This method allowed up to 77 times more adeno viruses to pass through the membrane into the muscle cell than before. With this new technique, which can also be used against other diseases, only the gene was transported which produces the easily detectable protein beta-galactosidase. Experiments to transfer the dystrophin gene are being prepared (*Kochanek, Cologne*).

**Transfer of naked genes:** For this technique, the genetic material DNA to be transferred is not built into viruses but into plasmids, small circular DNA structures without protein, that exist in bacteria where they mostly give rise to resistance against antibiotics. The advantage of this kind of gene transfer is that the plasmids do not contain any protein, only genetic material, *naked DNA*, so that no immune reaction develops against the vector material.

Experiments were made with plasmids containing marker or reporter genes for proteins that can be detected in the muscle tissue after staining or by light production so that the success of a

transfer experiment can easily be monitored.

When relatively large volumes of a solution of these plasmids were injected *under pressure* into the arteries of the limbs of rats and rhesus monkeys, the reporter genes beta-galactosidase and luciferase were transferred into up to 20 % of the muscle fibers after one single injection and into up to 40 % after repeated injections. The pressure was created by blocking the venous outflow from the limb for a short time. Experiments to transfer the dystrophin gene are currently being performed in mdx mice, monkeys, and GRMD dogs (*Wolff, Madison; Braun, Strasbourg*). In France, clinical studies with Duchenne boys have been started which are described in the section "Clinical studies".

**Experiments to overcome immunological problems:** As plasmids contain only DNA but not any proteins, gene transfer with such naked DNA allows the examination of potential problems with immune responses to newly created dystrophin without the interference of other proteins which are newly produced with the other methods of gene transfer using viral and cell vectors.

If the human dystrophin gene is introduced into the muscles of mdx mice, an immune response develops against the foreign dystrophin. This does not happen after the transfer of the dystrophin gene of mice, although normal dystrophin is *not present* in the muscles of these dystrophic mice. The lack of an immune response to mouse dystrophin may be due to the presence of the other forms of dystrophin. These results suggest that immune responses to dystrophin gene transfer may not be a particular problem in Duchenne patients, especially in those with point mutations that do not interfere with the synthesis of the other forms of dystrophin. Current experimental work is focussed on determining which of the other dystrophins must be present to allow immune tolerance to the appearance of new muscle type dystrophin after gene transfer (*Wells, London*).

## Experiments with stem cells

**Among the satellite cells and the myoblasts of the muscles, and also among the cells of the skin and the blood vessels, are stem cells which can form new muscles or regenerate them.**

Stem cells exist in many body tissues, e.g., also in skeletal muscles and in bone marrow. They

are non-specialized cells that can develop into some kinds of specialized cells, e.g. bone mar-

row stem cells into different types of blood cells and muscle stem cells into new muscle cells. These *pluripotent* cells are somatic or adult stem cells in contrast to embryonic stem cells which are *totipotent* and thus can develop into *all* kinds of body and germ cells. Stem cell research to find a therapy for Duchenne dystrophy uses with one exception only adult stem cells of experimental animals. Thus the ethical problems connected with the use of human embryonic stem cells can probably be avoided.

**Stem cells from skeletal muscles:** On the surface of the muscle cells are satellite cells, also called myogenic cells or myoblasts which, after an activation through several intermediate steps, can form new muscles or repair injured muscles. To determine whether such “back-up” cells are entirely or partly stem cells, satellite cells from the skeletal muscles of newborn mice were isolated. It was found that they consist of three different kinds, mainly the so-called EP and LP myogenic cells, which are already quite specialized. The third kind are *muscle derived stem cells*, MDSC. They are very rare, only one among 100,000 satellite cells is one such MDS cell. But these rare cells are *pluripotent*, that is, they can develop into muscle cells as well as into cells of the nerves and of the blood vessels for new muscles. And they can be cultured for a long time.

These MDS cells were multiplied in the laboratory, and then 400,000 of them in 25 microliters (a fortieth of a cubic centimeter) of liquid injected in one single portion into one muscle of living mdx mice. In order to see in what way these cells are different from the normal myogenic cells, the more specialized EP cells were also injected into mdx mice under the same conditions.

In the mice which were treated with the EP cells, 130 muscle cells at the injection site contained new dystrophin after 30 days, which however, after 90 days, had largely disappeared. In these mice, immune rejection by lymphocytes was observed which was probably responsible for the slow disappearance of the new dystrophin.

In contrast to these results, 1,500 muscle cells at the injection site of the mice treated with MDS cells contained new dystrophin, about 10 times as many as after the injection of EP cells. Even after 90 days, in practically all of these cells, the

new dystrophin was still present. In this case, there was *no* immune rejection although the receptor mice had not received any immune suppressive drugs.

As the muscle stem cells used in these experiments were isolated from *newborn* normal mice, this technique will probably not be applicable without modification with Duchenne children. They could, however, influence the recently resumed myoblast studies (*Huard*, Pittsburgh; *Wernig*, Bonn).

**Stem cells from bone marrow, muscles and skin:** At the end of the last decade, the first experiments with stem cells from bone marrow and with muscle satellite cells were performed. In all experiments, the stem cells were obtained from normal male mice with normal dystrophin genes and then injected into the tail vein of female mdx mice. The female mice were homozygous, that is, the dystrophin genes on *both* of their X chromosomes were mutated so that they could not produce any own dystrophin. The fate of the injected cells in the female mice could be followed by the detection of the male Y chromosome. The female mice had to be lethally irradiated with X rays to avoid an immune reaction. The transfer of the stem cells regenerated the bone marrow of the mice.

First, 10 to 50 million cells from the untreated bone marrow were injected. Three months later, dystrophin could be detected in up to 10 % of the muscle cells of the mice. Some of the dystrophin-positive cells contained Y chromosomes as proof that the bone marrow cells from the normal male mice came via the blood stream and had fused with the muscle cells of the mdx mice. They had brought with them the information for the normal dystrophin which was then used for the production of functional dystrophin. It was important to prove that the new dystrophin did not appear by reversion, that is, by spontaneous exon skipping which occurs to a small extent in mdx mice and also in Duchenne boys.

To find out which part of the bone marrow cells has the stem cell properties, a small side population, SP, could be separated from the original cell mixture by the FACS method, *fluorescence-activated cell sorting*. As these SP cells had stem cell properties, they were used for further experiments.

Three months after the injection of only 2,000 to 5,000 of these SP bone marrow cells, up to

4 % of the muscle cells of the mdx mice had functional dystrophin. In a similar way, SP muscle stem cells were isolated from muscle satellite cells, and 7,000 to 20,000 of them were injected into the blood stream of female mdx mice. After one month, 5 to 9 % of their muscle cells contained normal dystrophin and some also had Y chromosomes.

Now, in 2002/2003, these experiments were repeated and extended to stem cells which were obtained from skin. The advantages of an isolation from skin are that this tissue is more easily accessible than muscle tissue, and that the acceptor animals no longer have to be irradiated.

A small side population of cells was also obtained with the FACS technique from skin cells which had the same surface properties, markers, as the SP muscle stem cells. The percentages of SP cells were now 0.1 % from bone marrow cells, 0.7 % from muscle cells, and 1.2 % from skin cells. Three months after the injection of 6.000 to 50.000 of the SP skin cells, 2.3 % of the muscle cells of the female mice contained new dystrophin and also some Y chromosomes.

This experiment had proved that stem cells can be isolated from skin, and that, after application via the blood stream, they can form new muscle cells with normal dystrophin. This technique is not yet sufficiently effective to be of therapeutic significance. But after optimization, a Duchenne therapy might possibly be based on it (*Kunkel*, Boston).

**Activation of muscle stem cells:** Stem cells with specific surface structures which are located in *intact* muscles, did not develop into new muscle cells, they were *not myogenic*. But if the muscles were injured and the muscle cells regenerated themselves, then the number of muscle stem cells increased up to ten times. These cells were now *myogenic*, they developed into myoblasts and new muscle cells, as experiments with cell cultures and mice have shown. This activation was initiated by Wnt proteins, a family of signal proteins which apparently are produced by injured muscle cells and which also play a role during embryonic development (*Rudnicki*, Ottawa).

Some Wnt proteins have been isolated and characterized now. They are about 400 amino acids long and contain palmitic acid, a fatty acid which seems to be important for the transmittance of molecular signals (*Nusse*, Stanford).

**Mesoangioblasts, stem cells from blood vessels:** One half million of newly detected stem cells from blood vessels of fetal normal mice were delivered by one single injection into the artery of one hind leg of mice. These mice were missing one protein of the dystrophin complex, the alpha sarcoglycan which causes one of the many limb-girdle muscular dystrophies in humans. The injected cells migrated into the blood capillaries, passed through their cell walls, and from there into all muscles of the injected leg, especially into regenerating fibers.

For at least three months after the injection, new sarcoglycan was again present in almost normal quantities. The same was true for many of the other proteins of the dystrophin complexes which had also been missing. Also, there were no immune reactions. After three consecutive injections every 40 days, not only was the gene defect almost completely corrected, but also the muscle force in the treated leg was practically normalized again. Here as well, no immune problems were encountered.

In order to check whether this technique could possibly be used for a gene therapy, the mesoangioblasts from "sick" mice were isolated, and then the missing gene for alpha sarcoglycan was transferred with retroviruses into these defective stem cells. The systemic application of these ex-vivo treated cells led to the same positive results as found with the normal cells. However, retroviruses which insert themselves with the transported therapeutic gene into the chromosomes, should not be used in humans because of their risk of causing cancer.

For these experiments, the blood-vessel derived stem cells were isolated from unborn mice. But if it were also possible to isolate them from boys with Duchenne muscular dystrophy, these unexpected positive results would mean that a number of problems connected with the present gene transfer experiments could be avoided as, e.g., low effectivity, immune rejection, and the requirement of many injections into all muscles which can be reached.

Intact dystrophin genes would have to be transferred into these patient-derived cells by an ex-vivo procedure with the known vectors, then multiplied in the laboratory, and finally re-injected into the most important arteries of the child. Possibly, the treatment would have to be repeated after several months, therefore it is important

that these cells are not rejected by the immune system. And one of the most important advantages would be that, with this technique, all muscles could be reached, also the cardiac and respiratory muscles. It would be a *systemic* treatment with relatively few injections compared to other gene therapy approaches (*Cossu*, Milano).

**A stem cell experiment of nature:** At the age of one year, a Duchenne boy had received a bone marrow transplantation from his father because of another disease. As he could still walk at 14 years in spite of the reading-frame-shifting deletion of exon 45, it was assumed that stem cells from the bone marrow had provided the information for new dystrophin. Investigation of new bioprotic material, however, have now shown that the milder symptoms of the disease are due only to a small extent to the transplant, but mainly to a spontaneous additional deletion of exon 44, so that, in the mRNA, exon 46 follows directly after exon 43. This exon skipping normalizes the reading frame. Dystrophin can again be produced but is shorter and therefore causes the symptoms of a Becker dystrophy (see paragraph on exon skipping). Apparently, after 13 years, a bone marrow transplantation can contribute to some extent to a Duchenne therapy, however not sufficiently so to significantly change the disease (*Kunkel*, Boston).

**Cell therapy with myoblasts:** Muscles have their own stem cells, the satellite cells or myoblasts, which, during the development or repair of muscles, fuse together to form myotubes and then long muscle fibers. As the word “myoblast transfer” has been misused, one now often prefers to say *myogenic cells* instead of myoblasts.

In the years 1990 and 1991, extensive clinical studies with Duchenne boys were performed with myoblasts. This *myoblast transfer* technique had shown positive results in mdx-mice. The cells used contained the normal dystrophin gene because they were derived from a healthy donor, mostly from the father of the patient. They were applied in multiple injections at 0.5 centimeters distance into some muscles of Duchenne boys with the expectation that muscle cells with normal dystrophin would form. However, these experiments were not successful, because the transplanted cells did not migrate sufficiently inside the muscle, because there were immunological problems, and because almost all of the injected myogenic cells had died after a short time

(*Karpati*, Montreal, and others).

#### **New experiments to transplant myoblasts:**

But work on this technique continues in order to determine the reason why far less than 1 % of the transplanted myoblasts survived in the dystrophic muscles. Researchers are now trying to characterize these rare active cells and to find out how they can be isolated from the inactive cells. They are therefore looking for molecular signals, special substances in the muscle cells, which could activate the myoblasts (*Partridge*, London).

In earlier experiments, only two known drugs, cyclosporin A and cyclophosphamide were used to suppress the immune reactions in Duchenne patients. Now, other substances have been investigated in studies with monkeys. This has shown that the immune inhibitor FK506 alone or in combination with the inhibitor MMF avoids the rejection problems much better for several months. A greater application density with injection distances of only 1 millimeter and a higher number of transplanted cells contributed to the fact that in monkeys, up to 67 % of the muscle cells had taken up the myoblasts, they became *hybrid* muscle cells (*Tremblay*, Québec City).

A clinical study with the modified technique has been started in Canada (see section “Clinical trials”). Also in other laboratories, work is being done to improve this cell therapy technique. E.g., it was found that m144, a protein of the immune system, avoids the immediate death of the myoblasts after transplantation into mouse muscles (*Hodgetts*, Crawley, Australia)

#### **Experiments for an ex-vivo gene therapy:**

To avoid immune problems completely, experiments were performed to isolate the myoblasts from the patient himself and then, in cell culture, to transfer an intact dystrophin gene into the cells, before they are re-injected again. In preliminary experiments an *electroporation* technique was used to transfer the gene for a fluorescent marker protein into myoblasts in cell culture. This technique transiently permeabilized the membranes with a single electrical pulse, at e.g. 400 volts across a distance of 4 mm. Under optimal conditions, the marker gene could be transferred into up to 70 % of the myoblasts where it produced the fluorescent protein. The cells maintained their ability to fuse into myotubes, the next stage of muscle development (*Bernheim*, Geneva).

## Changing the genetic information (gene repair)

**Mutations of the dystrophin gene on the X chromosome are repaired with oligoribonucleotides.  
Entire exons are skipped in order to restore the reading frame.  
Premature stop codons can possibly be skipped.**

Experiments are performed with the aim not to introduce a functional dystrophin gene sequence into muscle cells, but to change the faulty genetic information, thus *repairing* the mutation. Such a technique would have four important advantages: (1) The risks of a virus-mediated gene transfer would be avoided, (2) not only the dystrophin of the skeletal muscles, but all other forms of dystrophin would also be repaired, (3) the tissue-specific regulation of the dystrophin production would be maintained, and (4), manufacturing the therapeutic agents, *oligonucleotides*, would probably be much easier and cheaper than making virus or plasmid vectors with the genetic material to be transported.

Oligonucleotides are short specific DNA or RNA sequences, which consist of a few bases connected to each other by ribose- or desoxyribose-phosphate bridges of the nucleic acid backbones. They can be manufactured automatically.

Three kinds of repair strategies are already being applied: (1) repairing the mutation on the level of the gene itself, (2) changing the genetic information during the splicing process of the pre-mRNA by *exon skipping*, and (3) ignoring a premature stop codon.

**Repairing the gene in the GRMD dog:** Attempts to repair point mutations at the gene level are made by using short *chimeric oligonucleotides*, which contain RNA on one strand and DNA on the other. The DNA strand of the chimeric oligonucleotide is perfectly complementary to the correct gene sequence at the point mutation site of the gene, while the RNA portion is perfectly complementary to the mutant sequence. This leads to paired quadruplex structures, four-fold strands, which are capable of correcting such a small mutation by activating the biological DNA repair mechanisms of the cell.

With this technique, a DNA and RNA oligonucleotide complementary to the splice-site mutation in the dystrophin gene of the dystrophic GRMD dog was injected into a 6-week old affected dog. The treated muscle showed: (1) evidence of restoration of the exon which was missing because of the mutation, (2) restoration of

the protein region encoded by the missing exon within the full-length dystrophin protein which was found localized correctly to the muscle membrane, and (3), most importantly, demonstration that the gene on the X chromosome was corrected. The repair of the mutation was sustained for almost a year in this dog (*Bartlett, Bethesda*).

**Gene repair in the mdx mouse:** In a similar experiment, the point mutation in exon 23 of myoblasts from mdx mice was repaired in-vitro, and these myoblasts then fused to myotubes which produced normal full-length dystrophin. Two weeks after one single injection of the oligonucleotides into the muscles of mdx mice, up to 2 % of the fibers around the injection site contained new dystrophin which was not revertant dystrophin, i.e. normal dystrophin after a spontaneous suppression of the mutation. This amount of new dystrophin remained stable for at least 10 weeks (*Rando, Palo Alto*).

**Exon skipping:** With this technique one tries to change a Duchenne mutation into a Becker mutation. This can be done by inducing the splicing mechanism, which cuts out the introns from the pre-messenger RNA, to also eliminate one *specific exon* after a point mutation or a deletion had shifted the reading frame and thus caused a premature stop codon. The aim of this approach is to restore the disturbed reading frame.

The gene with its mutation is not altered by *exon skipping*, but the messenger RNA, mRNA, no longer contains the information of the skipped exon. As the mRNA is shorter than normal, the dystrophin protein is also shorter, it contains fewer amino acids. If the missing amino acids are part of the central region of the dystrophin, they are often not essential, and the resulting shorter protein can still perform its stabilizing role of the muscle cell membrane. The result would be the change of the severe Duchenne symptoms into the much milder symptoms of Becker muscular dystrophy.

**Elimination of the mutation of the mdx mouse:** This strategy was used to by-pass the single base-pair defect, the point mutation in

exon 23 of the mdx mouse. An *antisense oligoribonucleotide*, consisting of 20 base pairs which were complementary to the RNA sequence of the pre-mRNA at the border region of exon 23 to intron 23, induced the splicing process to disregard the exon containing the mutation. The genetic information of exon 23, which codes for 71 amino acids in the rod domain, was thus *skipped* during the reading process.

This and the other investigated antisense-oligoribonucleotides were chemically modified, e.g., by protecting the normally free and sensitive OH-groups of the ribose units of the RNA by methyl groups (-CH<sub>3</sub>). About 5 micrograms (millionths of a gram) of these stabilized potential "gene drugs" were injected together with the detergent F127 into the leg muscles of living mdx mice. After two to four weeks, up to 20 % of the muscle fibers contained almost the normal amount of slightly shortened dystrophin. And, together with the other components of the dystrophin complex, it was located correctly on the cell membrane. The muscle force was significantly improved but not completely normalized. A repeated treatment increased the number of dystrophin-positive muscle fibers without the development of an immune rejection (*Wilton, Perth, Partridge, London*).

**Exon skipping in the mRNA of the human dystrophin:** Exon 45 of the dystrophin gene is the single most frequently deleted exon in boys with Duchenne dystrophy. This causes a frame shift in the mRNA and a premature stop codon leading to a truncated and non-functional dystrophin protein which is subsequently degraded in the muscle cells. However, if both exons 45 and 46 are missing *simultaneously*, the reading frame is not disturbed, not shifted, resulting in a shorter than normal dystrophin with 108 non-essential amino acids from the middle part of the protein missing. Patients with this specific deletion have the milder symptoms of Becker muscular dystrophy.

In-vitro experiments to specifically delete exon 46 from the dystrophin pre-mRNA in myotubes from mdx mice were successfully performed with four different antisense oligoribonucleotides complementary to a splicing regulatory sequence *within* exon 46. With these oligonucleotides, an *exon recognition sequence* (ERS) or an *exonic splice enhancer* (ESE) is blocked, structures which are necessary for the splicing proc-

ess, the joining of the exons in the mRNA.

Then, similar in-vitro experiments were performed with several antisense oligonucleotides specific to the analogous splice sites of the *human* exon 46. With one of them, consisting of 19 base pairs, it was possible to delete exon 46 from about 15 % of the dystrophin pre-mRNA in myotubes obtained from two Duchenne patients who had a deletion of exon 45. (The last page of this report shows the molecular details of this experiment.)

This percentage of shortened mRNA without exons 45 and 46 led to normal quantities of a shortened dystrophin in at least 75 % of the myotubes. After 16 hours, this new dystrophin could be detected in the cells, after 48 hours it had moved to the cell membrane and stayed there for at least one week. The re-appearance of the dystrophin also led to the restoration of the dystrophin complex in and under the muscle cell membrane. In the meantime, the reading frame could be corrected in in-vitro muscle preparations from six other patients who had different deletions and also a point mutation.

This technique is very specific, resulting only in the removal of the one targeted exon. With the same technique, it was possible to skip 18 other exons in muscle cell cultures. *Thus, this very promising strategy, proven in test-tube experiments, may possibly later convert the Duchenne mutations into Becker mutations of more than 65 % of patients with deletions.*

Ongoing studies investigate different methods for the transfer of the of the antisense oligoribonucleotides into a living organism. To this end, experiments with living mice are performed which, instead of their own dystrophin gene, have the human gene in their muscles which, in addition, has been changed by creating "human" deletions. Clinical trials on Duchenne boys with these methods can only be contemplated after these studies with "humanized" mice have given positive results.

As the structure of the dystrophin gene is known in all details, it is already possible to predict which exon would have to be skipped in order to restore the reading frame after a defined deletion or point mutation. At this time, such predictions can only be purely theoretical. It is not certain, whether the results obtained in cell culture or with mice, will be the same in Duchenne boys, and it is not certain either, whether,

in an individual case, the restored but shortened dystrophin will really lead to the symptoms of a Becker muscular dystrophy (*van Ommen, van Deutekom, Leiden*).

*Splice sites* are specific RNA sequences at the borders of exons and introns which are essential for the correct removal of the non-coding intron sequences from the pre-mRNA. This *pre-messenger RNA* is the first product of an active gene. After removal of the intron sequences by splicing, it becomes messenger RNA, mRNA, that moves to the ribosomes where it acts as the information transmitter for protein synthesis.

**Exon skipping with internally produced oligonucleotides:** For a new method of skipping exons, the antisense oligoribonucleotides do not have to be injected but are synthesized in the cell nucleus, where they are needed, after transfer of their gene. The intron sequences are cut out from the pre-mRNA in the cell nucleus by spliceosomes. They are complex structures consisting of several proteins and small RNAs, small nuclear = snRNAs, which recognize the exon-intron borders and which join the exons after splicing precisely and without shifting the reading frame.

Some of these very short RNAs are the U7-snRNAs. They bind to splice sites at special recognition sequences in the pre-mRNA, block the splicing of specific exons, and thus ensure that several proteins of different length are produced by one gene. The U7-snRNAs normally regulate the splicing processes of the mRNA of histones. Histones are proteins necessary for packaging the DNA in the chromosomes.

For this new method, the U7-snRNAs were genetically modified so that they no longer bound to the histone pre-mRNA but only to the splice sites in the region of exon 23 of the pre-mRNA of the mouse dystrophin. To achieve this, a gene coding for the modified U7-snRNA together with a creatine kinase enhancer was packed into plasmids as vectors and then, in a laboratory experiment, transferred into isolated myoblasts of the mouse. These cells then developed in the culture dish into muscle cells.

In the nuclei of the transgenic myoblasts, the transferred gene produced modified U7-snRNAs. They had new recognition sequences and bound now in front and behind exon 23 of the dystrophin pre-mRNA to sites important for the splicing process. Thus, these splice sites were blocked and exon 23 was removed together with the

introns during the splicing of the pre-mRNA (exon skipping). As the point mutation of the mdx mice is localized in exon 23, the removal of this exon had the consequence that the mdx muscle cells, which could not make any normal dystrophin, now produced a slightly shortened dystrophin protein which was localized at its correct site underneath the cell membrane.

The aim of this fundamental in-vitro gene experiment – outside of the living mouse – was to prove that the muscle cells themselves can produce the therapeutic antisense oligoribonucleotides.

For a human application, U7-snRNAs adapted to the individual mutation of the patient would have to be used, whose gene must then be transported with a gene therapeutic vector into the cell nuclei of the muscle fibers. An alternative would be an ex-vivo transfer transporting the U7-snRNA genes into satellite cells or other muscle stem cells and then injecting them into the blood stream or directly into the muscles. As the U7-snRNA genes are very short, this transfer would probably be easier than the transfer of the cDNA for the entire or the shortened dystrophin as is being tried with other methods (*Weis, Bern; Lochmüller, Munich*)

**Homologous recombination:** The point mutation in the dystrophin gene of mdx mice could be repaired in 15 to 20 % of isolated myoblasts by the addition of a DNA string of 603 base pairs whose sequence is the same as the sequence before and after the mutation site of exon 23 of the mice but not containing the C-to-T exchange of the mutation. Part of the new gene segment was exchanged against the corresponding, the homologous, in exon 23. This *short fragment homologous recombination*, SFHR, technique was then applied to isolated myoblasts from a Duchenne patient with a deletion of exon 13 (*Kapsa, Melbourne*).

**Ignoring a premature stop codon by antibiotics:** About 5 % of Duchenne boys have a point mutation in their dystrophin gene which changed an amino acid code word into one of the three stop codons, TGA, TAG and TAA, after which the synthesis of dystrophin stops prematurely.

*Gentamicin* is an antibiotic that causes the RNA translation mechanism in the ribosomes to ignore such a premature stop codon, i.e. to *read through* it. The normal stop codons, which are

protected by a special three-dimensional structure, will, however, be respected as before. In mdx-mice, up to 20 % of the normal amount of new and functional dystrophin has been obtained in this way. Gentamicin has the advantage of being a well known drug whose use as a possible therapy for Duchenne muscular dystrophy would not need long approval procedures (*Sweeney*, Philadelphia).

In order to confirm these positive results, two other studies with mdx mice were performed

which showed, however, that under similar conditions after a treatment with gentamicin *no* new dystrophin could be detected (*Karpati*, Montreal; *Lochmüller*, Munich).

Two clinical studies with gentamicin have been performed on Duchenne boys but have also not led to new dystrophin. Possibly the 14-day trial period was insufficient. Therefore, another and longer lasting clinical trial with 36 patients is now being performed (*Mendell*, Columbus).

## Replacement of dystrophin

**Utrophin is a muscular protein present in small amounts also in Duchenne patients. In larger quantities it could take over the function of dystrophin.**

**Upregulation of the utrophin gene:** Utrophin is a protein with a structure and function very similar to dystrophin. In humans, its gene is located on chromosome 6, it has 75 exons and is about one million base pairs long. The utrophin protein is about 7 % shorter than dystrophin. It is present in many body tissues, also in muscle, but there it is concentrated in regions where the motor nerves contact the muscle membrane. Before birth, the utrophin concentration in muscle is much higher than afterwards. This protein, though it is present only in small amounts, makes the Duchenne symptoms less severe than they would be if utrophin were also missing. In fact, mdx-mice whose utrophin gene was *knocked out* experimentally, which thus have neither dystrophin nor utrophin, have Duchenne-like symptoms and die early in contrast to “normal” mdx mice whose muscles hardly degenerate.

Experiments with mice have shown, that utrophin, if it is present in larger amounts, can replace dystrophin. The mice used were transgenic mice who contained utrophin mini genes in their germ line, introduced by a technique that cannot be used in humans. Other transgenic mice were raised which produced utrophin only when they were given the antibiotic tetracyclin in their drinking water. The increased amount of utrophin prevented the development of Duchenne symptoms, and this effect was more pronounced in newborn than in 10 or 30 day old mice.

For a possible Duchenne therapy, another strategy is followed, namely to increase the normally low amount of utrophin by *upregulation* of the activity of its gene. To achieve this, an activating substance is needed, which could well be

a known drug, or some other chemical or a naturally occurring substance.

During the last years, synthetic chemistry has developed methods to automatically produce thousands of partly unknown substances. Many of these substances are being tested in the laboratory, also automatically, on cell cultures from mdx mice for their ability to activate the gene of luciferase, which is preceded by the two promoters of the utrophin gene. The light producing enzyme luciferase from fireflies is easier to detect than utrophin. Every hit, i.e., every substance which shows at least a low activity in these preliminary tests, is further modified, and then, all these similar substances are tested first on muscle cell cultures, and, if they react positively, in living mdx mice, too, to see whether they can also upregulate the utrophin gene. Only after one or several convincingly active substances are found, would it be possible to start clinical studies with Duchenne patients in a few years (*Davies*, Oxford).

Such an activator of the utrophin gene could, if it is a small molecule, be applied via the blood stream from where it would reach all the muscles. And the immune system would recognize an additional amount of utrophin as a substance of its own because it is already present in small amounts in Duchenne boys. Therefore, no immune rejection should develop.

However, something that upregulates the utrophin gene, could also do the same with other genes. Thus, before such an activator is tested in children, it should be made certain that it does not produce any undesirable side effects.

### Other experiments to increase utrophin:

The transfer of a shortened utrophin gene with adenoviruses into *dystrophic dogs* led to utrophin which could take over the function of the missing dystrophin. --- *Glucocorticoides*, among them prednisone, can upregulate the utrophin gene to some extent. --- Such corticoides have also been isolated from *Chinese plant medicines* which traditionally are used against muscular dystrophy. --- Low grade chronic *inflammation* in muscles of mdx mice leads to a marked upregulation of utrophin on the membrane outside of the contacta with the motor nerves. --- The amino acid *L-arginine* can increase the amount of utrophin in mdx mice and alleviate significantly the dystrophic symptoms. The enzyme *nitric oxide synthase* uses arginine for the production of the biologically active gas nitric oxide. But nitric oxide is also active in other biological processes.

Arginine, therefore, cannot be used for a Duchenne therapy without further investigations. --- The small protein *heregulin* with which the motor nerves stimulate muscle development can also significantly alleviate the symptoms of mdx mice. --- One end of the utrophin mRNA is not translated into protein, but binds to structures at the contact regions of the nerves, thus restricting utrophin to these sites. If this binding could be prevented by a drug, it might become possible to distribute utrophin more evenly over the entire muscle membrane so that it can better replace dystrophin. --- Another form of utrophin, the very similar *B-utrophin* was recently identified in blood vessels. But only the "normal" A-utrophin is present in muscles and can partially compensate for dystrophin after upregulation of its gene.

## Other proteins

### The mutations of the dystrophin gene and the absence of dystrophin influence the activities of many other genes.

**Activities of thousands of muscle genes:** In order to measure simultaneously the activities of very many genes in one tissue sample in a single experiment, *gene arrays* are used. As the sequences of practically all human genes are known, short segments of thousands of genes can be produced automatically and applied by a robot in a certain pattern to a quartz chip a few square centimeters in size. If biochemically produced DNA copies of the mRNAs of all active genes are applied as an analysis sample to the chip, light points appear at those chip sites where there are complementary DNA sequences of these active genes. The light intensity of these points is automatically measured which then makes it possible to determine which genes are active and to what extent and which are not active.

With this *expression profiling*, several thousand genes in muscle samples were tested which came from normal and mdx mice, from healthy and Duchenne boys, from transgenic mice which neither had their own dystrophin nor utrophin, and also from other mice which instead had human dystrophin in their muscles.

The results showed that the absence of dystrophin causes the increase and the decrease of the activities of many muscle genes. A whole series of genes which are responsible for the ener-

gy production in muscle cells were less active in mice without dystrophin and utrophin, i.e. their muscles had an energy crisis which contributed to the degeneration of their dystrophic muscles. On the other hand, many genes necessary for the development and repair of muscles were increased in their activities, in some cases by a hundred times, i.e., they were upregulated by the disease process. Similar results were obtained with human muscle samples.

Further experiments showed that in mice many other genes were upregulated, genes which are involved in the development of surface structures, in the production of signal factors for protein synthesis, in the intensification of immune reactions, and in other processes responsible for the muscle dystrophic symptoms of mdx mice. In Duchenne patients, these changes were less pronounced, and in transgenic mice with *human* dystrophin, these gene activities were normal, because they no longer had a muscular dystrophy.

After these first results were obtained a few years ago, many more experiments with this new technique were performed to answer other questions of Duchenne research. Their and future results will help to understand the complex relationship between the many parts of the muscular

architecture and its changes if one of its most important components, dystrophin, is absent. This will open new avenues for the development of a Duchenne therapy (*van Ommen*, Leiden; *Kunkel*, Boston; *Hoffman*, Washington, and others).

**A dystrophic worm:** *Caenorhabditis elegans* is a 0.9 millimeter long transparent worm which is used extensively by gene researchers because all its 19,733 genes are known and also all its 959 body cells, 95 of which are muscle cells. Its muscles have a dystrophin similar to the dystrophin of humans, which can also have mutations causing dystrophic symptoms.

Individual genes were inactivated and also activated so that the dystrophic symptoms could be studied which were caused by the missing or increased gene activities. E.g., it could be shown that the upregulation of *dystrobrevin*, a very short form of dystrophin, slowed down the muscle degeneration significantly. Further investigations will contribute to the clarification of still unknown molecular relationships during the de-

velopment of muscular dystrophy (*Ségalat*, Lyon).

**Integrins and syntrophins:** *Integrins* are a family of proteins which are located in the muscle cell membrane. They are necessary for the fusion of myoblasts to myotubes and the development of myotubes to mature muscle cells. They also participate in the propagation of signals from cell to cell.

In mice without dystrophin and utrophin, the amount of one of these integrins was increased about twofold by gene transfer. This extended the life expectancy of the mice by threefold, and their dystrophic symptoms ameliorated significantly (*Kaufman*, Urbana).

The five known *syntrophins* are proteins that mediate the interaction of signalling proteins with the dystrophin and utrophin complexes at the muscle cell membrane. Understanding these effects in more detail may have consequences for a therapy with utrophin (*Froehner*, Seattle).

## Pharmacological strategies

### Corticosteroids and other drugs can ameliorate the symptoms of Duchenne muscular dystrophy without curing the disease itself.

As long as the efforts to transfer a functional dystrophin gene or to repair the damaged gene have not led to a cure, attempts are being made to at least alleviate the symptoms of Duchenne dystrophy by a drug treatment. There has recently been some success in this area.

**Myostatin:** The “blue-white” Belgian cattle have existed for about 200 years, they have 20 % more muscle meat than normal animals. Six years ago, it was found that these cows have a deletion of 11 base pairs in the gene for the protein *myostatin*. Transgenic mice without a *myostatin* gene are two to three times as heavy as normal mice, not because they have more muscle cells, but because their muscle cells are much larger. Myostatin is a signal protein, a kind of hormone, it consists of 375 amino acids and is necessary for limiting muscle mass.

This protein is produced in the muscle cells and their precursor cells, after which the protein is modified: two thirds of the amino acid chain is removed, and two of the remaining chains with 109 amino acids each form a double ring. This *active myostatin* inhibits the growth of the muscle cells by negatively influencing the genetic

regulation of the myogenic precursor cells. Other factors are also involved in optimizing muscle mass.

These facts led to the assumption that by blocking the activity of myostatin, the muscles of Duchenne boys could be made larger or at least their destruction reduced. Therefore, monoclonal antibodies were made, i.e., immune proteins that attach themselves very specifically only to myostatin and thus inactivate it. These antibodies were injected once a week under the diaphragm of mdx mice. After three months, the treated animals were 12 % heavier than control animals without treatment because their muscle mass had increased. In addition, they had a better muscle function, they could better cling to a rotating glass rod, their muscle degeneration had decreased and the CK activities were practically normal.

Further experiments are now being performed with mice. They have to be repeated with dystrophic dogs whose dystrophy is more similar to the human disease than the dystrophy of the mice. Only when the results are positive could clinical trials with Duchenne patients begin.

This treatment method would *not* be a cure of

Duchenne muscular dystrophy because the genetic cause of the disease would not be eliminated. But, compared with other methods, it would have advantages: no immune or toxicity problems, no genetic risks by viruses, and easy manufacturing of the drug. Pharmaceutical companies are already interested in this technique because increasing the muscle mass would also be important for older persons and people with other muscle-degrading diseases (*Khurana*, Philadelphia).

**Glucocorticoides:** The related corticoides *prednisone*, *prednisolone*, and *deflazacort* delay muscle degeneration, but the cause of this effect is not yet known. While part of their action likely involves their anti-inflammatory properties, other mechanisms of action are also possible. By analyzing the activities of more than one thousand genes in prednisone-treated mdx mice with the new micro array technique, it was found that about 5 % of the genes showed reduced or increased activities. Further analysis of the pattern of gene expression induced by glucocorticoids will help to understand the molecular mechanism of action of glucocorticoids in skeletal muscle. This might help to develop a more specific but less toxic treatment with these drugs (*Muntoni*, London).

**Creatine** can possibly also slow down muscle degeneration. It is a natural compound which is eaten in large quantities by athletes for enhancement of performance. Creatine when bound to phosphoric acid provides energy not only for muscle contraction but also for the removal of superfluous calcium, one of the causes of destruction of muscle cells. Experiments with mdx mice have shown that creatine supplementation can improve their disease symptoms, and this may provide a scientific basis for its use as supplementary therapy for Duchenne dystrophy (*Wallimann*, Zürich; *Rüegg*, Lausanne).

**Other pharmacological experiments:** Extracts of *green tea* in the food of mdx mice slowed down the degeneration of some of their muscles, possibly because this tea contains anti-oxidant substances. --- Transgenic mice were raised which produced in their muscles the *muscle insulin-like growth factor 1* (mIGF1) in relatively large amounts. This increased muscle mass by up to 40 %, and fibrosis as well as muscle degeneration were decreased and the regeneration significantly improved. --- *Leupeptin* consists of three partly modified amino acids. Combined with carnitine, it inhibits the enzyme calpain which destroys proteins in the muscle cells when, as in Duchenne dystrophy, calcium enters in an uncontrolled way. In experiments with monkeys and mice, muscle degeneration was significantly slowed down by leupeptin. --- The concentration of the enzyme *nitric oxide synthase* in the dystrophin complex of mdx mice is significantly reduced. Therefore, its product, the biologically active gas nitric oxide, can no longer fulfil its function. This contributes to muscle degeneration. Transgenic mdx mice with the normal amount of nitric oxide synthase showed reduced dystrophic symptoms. --- In mdx mice, the signal protein *JNK1* is activated and this contributes to the degeneration of muscle cells. The injection of adenoviruses carrying the gene for the natural protein *JIP1* inhibits the activity of *JNK1*, and this reduces muscle degeneration. --- The protein *galectin-1* participates in the processes leading to new and regenerated muscle tissue. Fibroblasts obtained from the skin of newborn mice develop into muscle cells with dystrophin if they grow in a cell culture which contains galectin-1. Such fibroblasts could be easily collected from a Duchenne patient, transformed into muscle cells with galectin-1, then genetically modified to synthesize normal dystrophin, and finally transplanted back into the patient.

### Clinical studies with Duchenne patients

**The first gene transfer trial and a new trial with myoblasts have begun.**

**Corticosteroids, creatine, and other chemical substances are studied.**

**A large study with prednisone together with cyclosporin is being prepared.**

Clinical studies with Duchenne boys will be more and more necessary in view of the increasingly positive results with experimental animals. These studies with humans will have to be performed in several steps, of which the first one,

*phase I*, will already take several years to prove that the new treatment will not be accompanied by unacceptable side effects. Only afterwards can further studies be started with sick children to ascertain whether the treatment really im-

proves or maintains the muscle force, *phase II*, and what the optimal dosage will be, *phase III*.

All these studies have to be performed *doubt-blind*, i.e., only about half of the patients receives the substance to be tested whereas the other half receives an inactive compound, a placebo. And neither the patients nor the researchers are allowed to know which patient belongs to which group before the trial is completed, the code is broken and the results are analyzed. These studies and the approval procedures are time consuming, they take many years, and are expensive to perform.

**Transfer of the dystrophin gene with plasmids:** The first phase of the first gene transfer experiment with Duchenne patients has been completed at the beginning of 2003 and the results reported in June 2003. The biotechnology company *Transgène* in Strasbourg together with the French muscular dystrophy association *AFM* started to prepare this gene therapy approach in 1995. The permission for this first human trial was given by the French authorities in November 1999, and the first injections of the vectors were performed in September 2000 at the *Hôpital de la Pitié Salpêtrière* in Paris.

The 9 participating boys were all older than 15 years so that they could give their *informed consent*. They did not derive any clinical benefit from this treatment, *it was not yet a therapy*.

After several gene transfer methods were tested on dystrophic mice and dogs, it was decided to use the entire cDNA of the gene for the full-length dystrophin placed in a *plasmid* as vector together with a strong promoter from a virus. Plasmids have the advantage of not containing any protein and thus should not cause an immune reaction. The therapeutic gene to be transported has no protein either, it is pure or *naked DNA*.

In further preliminary experiments with muscle cell cultures and mice, it was shown that this vector construction led to the appearance of new dystrophin at its correct place underneath the muscle cell membrane of the animals, that it restored the dystrophin-glycoprotein complex, and that it prolonged the life of the cells.

The aim of this clinical study with Duchenne patients was to show that the procedure is safe, i.e., that it does not lead to an immune reaction or an inflammation, and that new and normal dystrophin appears at the correct places in those fibers of the muscle which had received the

plasmid vector.

A solution containing 0.2 mg of plasmids with 10 trillion ( $10 \times 10^{12}$ ) copies of the dystrophin gene was injected into one muscle of the forearm of the first three patients. This is a very small amount of genetic material compared to similar experiments in animals. The next three patients received one dose of 0.6 mg and the last three two doses of 0.6 mg two weeks apart. The safety of the patients was the main concern, therefore, any one patient was treated only when it was certain that the previous one treated did not show any signs of immune intolerance.

Three weeks after the injections, the treated muscle area of about 0.5 cubic centimeters was extracted by biopsy and checked for the presence of dystrophin. In three out of six boys in the first two groups and in all three boys in the third group, new dystrophin appeared in less than 1 % to more than 25 % of the muscle fibers around the injection sites. There were no signs of an immune reaction, neither to the plasmid nor to the newly produced dystrophin. This answered the question of a phase-I study: *Gene transfer with naked DNA is a safe procedure*. It could, after amplification, become a therapeutic method because it is known from animal experiments that dystrophin production in about 20 % of muscle fibers would improve muscle function significantly.

The French scientists are now working with the team of *Jon Wolff* in Madison in the United States, who injected similar plasmid constructions with genes of a marker protein into the blood stream of limbs of rats, dogs, and monkeys *under pressure*. Afterwards, up to 40 % of the muscle fibers contained the transferred marker protein.

The next step will be to apply this arterial delivery procedure in Duchenne boys, probably in a clinical trial starting in 2004. At that stage, it is planned to treat, again for safety reasons, only a small foot muscle. Should the results be positive, this method will be tried on entire arm or leg muscles. This could be planned for 2006.

Afterwards, respiratory and cardiac muscles could be targeted. For safety reasons, it is impossible to proceed faster, because it would be a catastrophe should severe side effects or any other undesired event happen which would lead to the interruption of this and other gene therapy experiments (*Braun*, Strasbourg).

**Clinical studies with myoblasts** (myoblast transfer): At the beginning of 2003, a clinical phase-I trial with 5 to 15 year old Duchenne boys with deletions has started in Québec City in Canada. It should answer the question whether the transfer of normal myoblasts under modified conditions is safe, i.e., that it does not create an immune rejection or inflammation, and whether new dystrophin appears after the treatment.

The difference with regard to the unsuccessful experiments performed in 1990 is that the much more effective immune inhibitor FK506 (Tacrolimus) A is used instead of cyclosporin, that not, as before, 60 to 90 million cells are injected into the entire biceps muscle, but 30 million cells into the foot muscle tibialis anterior by multiple applications at a distance of one millimeter from each other into a muscle volume of only one cubic centimeter. An improvement of the muscle function is not expected. As in the French trial, the participants will have *no therapeutic benefit* from these injections.

One month after the treatment and after a biopsy it will be determined whether normal dystrophin DNA, mRNA and protein have appeared and whether there were any immune reactions. Three patients have been treated until July 2003, the entire trial should be finished before the end of 2003.

If the results are positive, the trial will continue with a phase-II study, during which myoblasts will be transferred into the entire biceps muscle. Then, during the following two years, the muscle force will be measured which, as is hoped, will have possibly increased or at least remained unchanged (*Trembley*, Québec City).

**Prednisone and deflazacort:** Sixteen clinical studies worldwide had proven the ability of glucocorticoides, cortisone derivatives, especially of *prednisone*, to maintain the muscle force of Duchenne boys. A few years ago, there were indications that the new corticoid *deflazacort*, which is related to prednisone, would act similarly but would show fewer side effects.

From 1992 to 1997, a study with the participation of 14 German muscle centers was performed in which the muscle maintaining effects of these two drugs were compared with the well documented natural history of the disease. The doses were 0.75 mg per kg body weight and day for prednisone and 0.9 mg per kg and day for deflazacort. The result was that both drugs can

maintain the muscle force for at least two to three years and, in isolated cases, prolong walking ability until about the 14<sup>th</sup> year. The most important side effect of prednisone was weight gain in about 20 % of the patients. With deflazacort, slight cataracts, clouding of the eye lens, were more frequent than with prednisone. Both drugs had a growth retarding effect, other side effects were insignificant. After stopping the medication, muscle degeneration and normal growth resumed again.

The results of the study do not yet allow a decision on the best time to begin the treatment, e.g. before five years of age. Children who wish to begin this treatment should do this in the setting of a well documented study in order to obtain more information and, because of the side effects, to guarantee the necessary controls. The study in Germany is continued as an open trial with long-term documentation which includes data for some patients who were treated for more than seven years (*Reitter*, Mainz).

**Prednisolone:** In the United Kingdom, a large long-term trial had been prepared which should have tested whether *prednisolone* is able to prolong walking ability and to improve life quality. An intermittent treatment, 10 days with and 10 days without medication had been planned. Because financing could not be obtained, it is now only possible to conduct an open study, not double-blind, with the aim of documenting efficacy and side effects (*Muntoni*, London).

**Prednisone combined with cyclosporin A:** In Germany, a clinical study is being prepared to start at the end of 2003 which should answer two questions: Can *cyclosporin A* alone increase muscle force over a short time? And can a combination of cyclosporin A and *prednisone* better reduce the loss of force over the long term than prednisone alone, when prednisone is given intermittently, i.e., in a cycle of 10 days with and 10 days without therapy? As immune processes play a role in Duchenne muscular dystrophy, earlier experiments have shown that immunosuppressant drugs such as cyclosporin A may delay muscle degeneration.

In order to obtain reliable results, at least 150 Duchenne patients should participate who are unequivocally diagnosed, who are older than six years, and who can still walk alone for 50 meters. During the first phase lasting three months, all children will receive, in a double-blind proce-

3.5 to 4 mg/kg/day cyclosporin A alone or galactose, milk sugar, as placebo. During the second phase lasting 12 months, all children will receive *in addition* 0.75 mg/kg/day prednisone for 10 days followed by 10 days without prednisone. Eight German muscle centers will perform this trial (*Korinthenberg*, Freiburg).

**Creatine:** A double-blind study with 8 Duchenne, 10 Becker, and 18 patients with other muscle diseases showed after 8 weeks with daily doses of 5 grams creatine monohydrate for children and 10 grams for adults a slight but significant beneficial short-term effect on muscle strength and performance without any side effects. More clinical studies have to be made before creatine can be recommended as a long-term muscle preserving medication for Duchenne boys (*Walter*, Munich).

In Canada, a trial to determine the effect of creatine has started with 40 Duchenne boys. --- In Belgium, creatine was tested for three months on 12 Duchenne and 3 Becker patients with the result of a slightly increased muscle force.

**Oxandrolone**, an anabolic steroid sometimes used by athletes, was tried in a study with 51 Duchenne boys. However, the small improvement of muscle strength found does not justify its use as a drug instead of prednisone or deflazacort (*Fenichel*, Nashville).

**International clinical tests:** The *Cooperative International Neuromuscular Research Group*, CINRG, in Washington, a cooperation of laboratories in the US, in Canada, Belgium, Argentina, Australia, and India, organizes clinical trials on Duchenne boys with substances some of which have been selected from 45 substances which had shown positive results when many com-

pounds had been tested in a large screening experiment with mice.

**Albuterol**, an asthma drug, had shown a significant increase of muscle strength (about 8 %) and very few side effects in a preliminary trial with 10 Duchenne boys. In August 2002, a double blind study has started with 25 – 30 boys to last about 9 months.

A trial with **coenzyme Q10** started in September 2001 with 15 boys which also regularly receive deflazacort or prednisone. Another trial is planned with patients in wheelchairs.

**Oxatomide**, an antihistamine, is being tested in a 9 months trial with 15 Duchenne boys.

**Pentoxifylline** interferes with the immune system and thus reduces inflammation and fibrosis. A trial started in February 2003 and will last 15 months.

A trial lasting 15 months has started in January 2003 to compare the positive and negative effects of **prednisone** when it is given every day or only on two days each week at a higher dosage.

A trial with 54 Duchenne boys was performed for 6 months ending in March 2003 in which either **creatine** or **glutamine** was tested in a double blind study. The results are being evaluated.

Trials with three other substances, **taurine**, **carnitine**, and **nicotinic acid**, are planned.

For the documentation and supervision of these studies, standardized control methods have been developed to measure not only muscle functions but also other parameters such as the quality of life. Some of these methods are being modified so that they can be also used for very young and older patients (*Escolar*, Washington).

## When will there be a therapy?

Duchenne muscular dystrophy has always been with man and all animals with skeletal muscles. Its clinical symptoms were described quite correctly for the first time in 1851 by the English doctor *Edward Meryon*. But, it got its name after the French physician *Duchenne de Boulogne*, who, in 1861, described not only its symptoms but also the muscular changes, its histology.

From its mode of inheritance, it was known at the beginning of the 20<sup>th</sup> century that a defect on the X chromosome is responsible for the disease. But only in 1986 was the gene itself, the *dystro-*

*phin* gene, identified (*Kunkel*, Boston) and shortly afterwards the protein *dystrophin* characterized (*Hoffman*, Washington), which is missing in Duchenne boys. The fast pace of genetic research gave rise to the hope that it would soon be possible to replace or repair the gene and thus cure the disease.

However, this optimism was premature. The first studies in 1991 with the *myoblast transfer* showed that this technique, which looked promising in mice, was *ineffective* in Duchenne boys. Now, 17 years after the detection of the gene,

there is still no therapy for Duchenne muscular dystrophy. As this report shows, research work is being done with many different methods which are tested in mice, dogs, and monkeys, and some already in Duchenne boys. But these studies are time consuming, and the approval of a treatment will take additional years.

However, there are examples of treatments that reach patients very quickly, such as *Gleevec*, a drug which was approved in 2001 within a few months without examining all side effects after it was shown that it could cure about 90 % of patients with the blood cancer chronic myeloid leukemia.

All the research results must be considered

and many more obtained before it is possible to make any prediction of how long it will take until a safe and effective treatment is ready for children with Duchenne muscular dystrophy everywhere in the world. The answer to this question is the most important one for the parents and their sick sons. It will probably still take many years, until Duchenne muscular dystrophy will be defeated. This is less than what has been hoped for, that is the negative side of this difficult problem, the positive is that hundreds of capable and dedicated researchers in many laboratories around the world are working on a cure: therefore, *it is certain that an effective treatment will be there, sooner or later.*

### Scientists mentioned in this report

Only the scientists mentioned in this report are listed with their abbreviated addresses and without any titles. Most of them are professors and all have an MD or PhD. Further information including many original publications, which contain the names of all members of a research team, can be obtained from the author of this report.

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**This report will be updated from time to time with new research results. Those who wish to receive the updates and the 2004 version of the report should send their e-mail address to Dr. Scheuerbrandt.**

**The report can be seen on the internet at <http://www.duchenne-research.com>, also in German, French, Spanish, and Italian.**

## Exon Skipping, an Example

Here, the molecular details of skipping exon 46 are explained with which the Duchenne muscular dystrophy caused by the exon 45 deletion is changed to a Becker muscular dystrophy.

Part of the base sequence of exons 45 and 46 of the mRNA of the dystrophin gene is shown as well as the end of exon 44 and the beginning of exon 47. In exon 45, 50 triplets are not shown and 30 in exon 46. Below each triplet (codon), the abbreviated name of the amino acid is shown according to the genetic code. The triplets follow each other without spaces, the hyphens indicate here only the reading frame and the vertical lines the borders of the exons. The exon-skipping “therapeutic” oligoribonucleotide attaches itself to the underlined 19 bases in exon 46 of the pre-mRNA. The three bases of the hidden stop signal are also underlined. Exon 45 ends after the second base of the last triplet, which then is completed to AGG by the first base of exon 46 (-AGG-AG | G-CUA-).

```

End Exon 44 | Start Exon 45           End Exon 45 | Start Exon 46
-UGG-UAU-CUU-AAG | GAA-CUC-CAG-GAU---AGA-AAA-AAG-AG | G-CUA-GAA-GAA-
  trp tyr leu lys | glu leu gln asp   arg lys lys arg|   leu glu glu
  
```

hidden stop code

antisense oligoribonucleotide

```

GUC-GUU-GAU-UUU-UUU-UUC-G           End Exon 46 |
--AAU-GAA-UUU---AAA-GAG-CAG-CAA-CUA-AAA-GAA-AAG-CUU-GAG-CAA-GUC-AAG |
  asn glu phe   lys glu gln gln leu lys glu lys leu glu gln val lys |
  
```

| **Start Exon 47**

```

| UUA-CUG-GUG-GAA-GAG-UUG---
| leu leu val glu glu leu
  
```

If only exon 45 is missing in the mRNA, the reading frame in exon 46 is shifted one nucleotide to the left, exon 46 then starts

```

instead of      | G-CUA-GAA-GAA-C   with      | GCU-AGA-AGA-ACA
                |   leu glu glu     |   ala arg arg thr
  
```

with the consequence that 16 incorrect amino acids are incorporated into the dystrophin until finally a premature stop signal UGA is reached which was hidden before (-AAU-GAA-UUU- is changed to -AAA-UGA-AUU-, the hidden UGA is underlined above). The protein synthesis is interrupted prematurely, the dystrophin remains incomplete, and *Duchenne muscular dystrophy* develops. After the deletion of exon 45, exon 44 is followed directly by exon 46:

```

End Exon 44 | Start Exon 46
-UGG-UAU-CUU-AAG | GCU-AGA-AGA-ACA---AGA-UUU-AAA-UGA-AUU-UGU-UUU-AUG-
  trp tyr leu lys | ala arg arg thr   arg phe lys STOP!
  
```

If in addition to the missing exon 45, exon 46 is also removed, the reading frame is not disturbed, there is no premature stop signal, but 108 amino acids are missing in the central part of the dystrophin, which, however, is still partly functional. This changes the severe Duchenne muscular dystrophy in the much less severe Becker muscular dystrophy.

```

End Exon 44 | Start Exon 47
---UAC-AAA-UGG-UAU-CUU-AAG | UUA-CUG-GUG-GAA-GAG-UUG---
  tyr lys trp tyr leu lys | leu leu val glu glu leu
  
```