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MOLECULAR GENETICS OF CHRONIC GRANULOMATOUS DISEASE

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INTRODUCTION

The work presented in this thesis is concerning some of the aspects of the characterization of the molecular defects responsible of a rare genetic disease (chronic granulomatous disease) affecting three patients at the Children Hospital in Trieste. This work was started as the first step of a research protocol aiming at the development of gene therapy techniques for these patients (i.e. transfer of the correct gene in bone marrow cells of these patients). For this reason, some of the techniques utilized were specifically set up not only for the characterization of the defects of these patients, but also as a tool for further monitoring the success of gene transfer.

A description of the clinical features of chronic granulomatous disease, of the biochemistry and genetics of the metabolic system underlying the defect will be presented in the following sections of the *Introduction*. The reasons why this disease was chosen for a gene therapy approach will be presented in the *Results* section.

❶ Clinical features of Chronic Granulomatous Disease

Chronic granulomatous disease (CGD) is an inherited hematological disorder, described for the first time nearly 30 years ago (for a review, see [Smith, 1991]).This is a rare condition characterized by the inability of phagocytic cells (neutrophils, monocytes, macrophages, and eosinophils) to produce antimicrobial oxidants. Affected individuals, mostly pediatric patients, develop recurrent suppurative infections resistant to common antibiotic therapies. The clinical signs are usually heterogeneous, as they derive from the localization of bacterial and fungal infections with the formation of granulomas. Different clinical pictures are possible, usually characterized by severe infections in infancy followed by two or three recurring episodes per year; patients in which the first infection appears in the

late childhood, however, are not uncommon; these latter individuals may then be symptom free for years before the next infection supervenes. It is likely that the wide variety of clinical onset and evolution of the disease is linked to the vast heterogeneity of genetic defects of the patients. The prognosis of the disease is obviously dependent on the severity, the number of the recurring infections and the beginning age of infective episodes.

Although formation of granulomas occurs in any district of body, they are commonly found in the liver, in the lungs, in lymph nodes and in the subcutaneous tissues [Smith, 1991]. In the lungs the infections are usually responsible of focal necrosis with subsequent fibrosis, bronchiectasis and postnecrotic emphysema with serious alteration of the respiratory capacity. Also damage to the liver is very serious, consisting in postinflammatory fibrosis leading to alteration of hepatic functions. A striking features is that the granulomas are only partially able to delimit the infection, while, on the contrary, they lead to serious tissue damage. The persistence of microorganisms in the tissues, often within the phagosomal vacuoles of the neutrophils or macrophages, constitutes a stimulus to a chronic inflammatory state: as a consequence, the granulomas become bigger and bigger reaching massive proportions.

Any bacterial strain can be isolated from the granulomas, and often infections are due to microorganism with a low grade pathogenicity. Fungal infections, that occurs at least once in the life of all patients, is mostly due to aspergillus and candida. It is likely that these infections develop as a consequence of antibacterial management that implies an aggressive treatment of each infectious episode and a preventive therapy.

Although in the last years the more aggressive therapeutically approach improved the clinical picture of many affected individuals, CGD remains a serious disease able to cause high infant mortality and infirmity.

❷ Biochemistry of CGD

When phagocytes are activated by the contact with microorganisms or their products a complex metabolic transformation takes place. The consumption of oxygen increases rapidly; soon thereafter the concentration of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) increases greatly in the surrounding medium. These events represent the so called *respiratory burst*. The generation of these strong oxidants is the result of the activation of an otherwise dormant membrane-bound enzymatic system that catalyzes the one-electron reduction of oxygen to O_2^- [Orkin, 1989]

The cellular defect in chronic granulomatous disease is due to the inability of phagocytic cells (neutrophils and macrophages) to produce superoxide and hydrogen peroxide. These cells, therefore, are unable to kill microorganisms after phagocitosis. In fact, although this is not the only system adopted by these cells to kill ingested microorganisms, its impairment is sufficient to alter substantially this function.

The superoxide anion (O_2^-) production system is based on a respiratory chain localized on the cytoplasmic membrane of phagocytic cells. This respiratory chain utilizes NADPH produced by the monophosphate hexose shunt to reduce a molecule of FAD to FADH, which in turn gets oxidized by reducing a cytocrome b. This cytocrome interacts with a molecule of O_2, reducing it to superoxide anion (O_2^-) [Stossel, 1987]. The cytocrome b is called b^{-245} because of its midpoint potential, i.e. the point at which it is balanced between oxidation and reduction. Since it displays an absorption band at 558 nm, it has alternatively been called b^{558}. This cytocrome is the non-functional molecule in CGD.

Inside the phagocytosis vacuoles, superoxide anion is highly reactive with

many macromolecules of the microorganisms; furthermore, the enzyme superoxide-dismutase catalyzes the production of H_2O_2 according to the reaction:

$$2O_2 + 2H \rightarrow H_2O_2 + O_2;$$

lactoferrin, in turn, catalyzes the reaction:

$$O_2^- + H_2O_2 \rightarrow O_2 + OH^- + OH^\bullet \text{ (Haber-Weiss reaction).}$$

Also $OH^\bullet$, presenting an unpaired electron, is highly reactive with the macromolecules of the microorganism.

In order to clearly understand the molecular defects leading to CGD, it is necessary to know in detail the structure of cytocrome b^{558} together with the interactions of this molecule with other cytoplasmic proteins. These proteins are schematically presented in Figure 1. The cytocrome b^{558} is constituted by two subunits of 91 Kd (gp91) and 22 Kd (p22) [Segal, 1988]. These two proteins are embedded in the cell membrane. In the cytosol, two other proteins of 47 Kd [Rodaway, 1990] and 67 Kd [Volpp, 1988], respectively, are present, whose function is essential for the cytocrome. A 22 Kd *ras*-like G protein, termed *rap*, is intimately associated with the cytocrome and may also be an obligatory component of the oxidase [Quinn, 1989]. An additional component (N) is present in the cytosol, constituting the NADPH-binding protein.

After activation of phagocytic cells, phosphorylation of the 47 Kd subunit occurs with the subsequent migration of the two cytosolic components where the cytocrome b^{558} is located [Smith, 1991]. After assembly of all the subunits, production of O_2^- starts, together with further phosphorylation of the 47 Kd subunit.

The mRNA transcript for the gp91 subunit is expressed in a highly lineage-specific manner [Royer-Pokora, 1986]. In neutrophils, monocyte/macrophages, and presumably eosinophils, the mRNA is particularly abundant, perhaps accounting for 0.1% or more of cellular mRNAs [Orkin, 1989]. A low level of mRNA is also

detectable in normal EBV-transformed B-cells, which are reported to have low level of NADPH-oxidase activity [Volkman, 1984]. On the contrary of the pattern of expression of the gp91 mRNA, that mirrors the distribution of the neutrophil cytocrome b and oxidase activity in various cell types, expression of the mRNA for p22 is not cell type-specific, being present in a wide variety of cell lines in abundance comparable to that found in phagocytic cells. Despite of this constitutive expression at the mRNA level, however, non phagocytic cells contain very little if any stable p22 polypeptide. This observation seems most compatible with a model in which the p22 subunit may be unstable intracellularly in the absence of the gp91 chain. Therefore, it can be argued that gp91 may serve a critical role in directing p22 into the functional membrane complex [Parkos, 1988].

❸ Genetic aspects of CGD

The complexity of the NADPH-oxidase and the multiplicity of its subunits predicts that CGD has to be a heterogeneous disorder. Of the four major proteins identified as involved in the production of O_2^- (gp91, p22, p47, p67) three are coded by autosomal genes, while gp91 is coded by a gene located on the X chromosome. Therefore, the transmission of CGD occurs either as an X-linked or an autosomal recessive character. About 65% of the patients show X-linked, and 35% autosomal recessive transmission [Curnutte, 1987].

Some of the properties of these polypeptides are reported in Table I

<u>X-linked transmission</u>

Within the X-linked forms, the most frequent consists of patients with complete absence of gp91 with subsequent absence of all the cytocrome b_{588} complex [Smith, 1991]. Although in most of the cases studied thus far the genetic defect is associated with a deficiency of the mRNA transcript for the 91 Kd subunit [Ohno, 1986], cases where the amounts of mRNA for gp91 fall within the range of

normal individuals have also been described [Bolscher, 1991]. On Southern blot analysis the genomic DNA usually appears grossly normal with no detectable deletions or rearrangements. Presumably, point mutations are responsible for the defect [Ohno, 1986].

A striking features is that characteristic point mutations have not been described so far. Substitutions, point deletions and insertions can occur all over the coding region of the gp 91 gene. The complete deletions of the whole gene for gp91 is very rare. This deletion usually occurs in patients with complex phenotypes, including McLeod syndrome, Duchenne muscular dystrophy and retinitis pigmentosa, suggesting of a vast deletion on chromosome X [Frey, 1988]. Also infrequent are the partial deletions of the gene [Royer-Pokora, 1986].

Independently from the genetic defect, the oxidative capacity of phagocytic cells of these patients, as evaluated either by the NBT test or the direct measurement of O_2^- production, is highly depressed [Curnutte, 1987], resulting in the most severe form of the disease.

Patients in which the protein is present but not functional, or patients with only a partial amount of the protein and a residual capacity of oxidative burst are less frequent.

Autosomal recessive forms

Among the autosomal recessive forms, the most important is due to the absence of the 47 Kd protein. In this case, the spectrum of absorbance of the cytocrome b^{558} is absolutely normal, but production of O_2^- is deeply impaired. Patients with defects of the cytosolic proteins usually present a milder disease as compared with patients lacking the membrane proteins [Weening, 1985].

The molecular defects responsible of the impairments of the 47 and 67 Kda proteins have not been investigated extensively; it was reported that in cases

where the 47 Kd protein is involved, the length and the amount of its messenger are normal [Lomax, 1989].

The absence of the 22 Kd protein mirrors the biological situation related with the absence of the 91 Kd protein. In both cases the cytocrome b^{558} is absent. Point mutations are the major causes of this autosomal defect [Orkin, 1989].

RESULTS

The experimental results described in these thesis were obtained from samples from two patients (A.G. and A.Z.) affected by CGD and followed at the Children Hospital *Burlo Garofolo* in Trieste. Both the patients are males and are affected by the X-linked form of the disease and have a long story of recurrent infections badly controlled by antimicrobial therapy. A brother of patient A.G., also affected by CGD died in 1991 after a long history of the disease. More recently, samples from another patient (N.B.) were obtained. This patient is also affected by the X-linked form of the disease, but his disease is milder. He is followed at the Ospedale Generale Regionale *Miulli* in Bari.

Due to the clear X-linked transmission of the disease, resulting both from clinical data and from NBT test on other members of the family, the attention was concentrated on the gene of the gp91 subunit of cytocrome b^{558} , that is encoded by the X chromosome.

❶ Southern blotting with DNA from lymphoblastoid cell lines from CGD patients

In order to evaluate the presence of gross deletions within the gene for gp91, Southern blot analysis was performed with DNA from two patients (A.G. and A.Z.) extracted from established lymphoblastoid B cell lines (see below). A DNA fragment containing the whole coding sequence of the transcript of the gene was used as probe for the hybridization. The results of the hybridization are shown in Figure 2. DNA samples from the two patients and a sample from a normal individual (N) were digested with *Hind III* and *Eco RI*, the resulting fragments resolved by gel electrophoresis, transferred to a nylon membrane and hybridized. It is apparent from Figure 2 that the hybridization pattern for both enzymes is indistinguishable for either the two patients and the normal individual.

As stated above, only the coding portion of the cDNA (~1.6 kb) was

available to us as hybridization probe. As a consequence, only a portion of the gp91 gene, totally spanning about 30 Kb [Smith, 1991], was explored by this hybridization, corresponding to about 7.5 Kb for the *Hind III* digestion and 14 Kb for the *Eco RI* digestion. Therefore, deletions in intronic regions not revealed by the probe could not be detected by this analysis. Nevertheless, gross deletions of the genomic locus or selective deletions of the coding sequence of the gene are excluded in these patients.

❷ Development of a competitive RT-PCR method for quantitation of low abundance mRNAs

The next approach in the analysis of the molecular defects responsible of the disease in the two patients was the study of the transcription pattern of the gene. This task was indeed quite problematic, since the gp91 gene is transcribed exclusively in myeloid cells [Smith, 1991] and at a low abundance. Therefore, quantitation of RNA by Northern blotting or RNA protection methods results quite difficult to perform. Furthermore, the characterization of the molecular defect in these patients is the first step of a long term project aiming at a gene therapy approach, where evaluation of the efficiency of gene transfer and expression in transfected bone marrow colonies is essential, and where, again, the availability of mRNA is very low.

For all these reasons, we decided to set up a system to quantitatively analyze the amounts of low abundance RNAs. This chapter describes the general set up of the method, which has extensive applicability to several other areas where precise quantitation of RNA is needed (such as quantitation of transcription and quantitation of viral RNAs). The following chapter describes the application of this method to the quantitation of the transcripts for gp91 in patients and normal individuals.

A method for quantitative PCR for DNA samples has already been

described in our laboratory [Diviacco, 1992]. We have now applied this method to the quantitation of RNA. The principle of the method consists in the co-amplification of the RNA sample to be quantified with different known amounts of a competitor RNA molecule that contains the same sequence (including primer recognition sites) of the target molecule except for a small insertion of 20 nt in the middle. The two RNA species compete for amplification, and any predictable and unpredictable variable that affects amplification have the same effect on both. Therefore, at the end of the reaction, the amplification products for the two species can be simply resolved by gel electrophoresis, and, independently of the total yield of the reaction, of the presence of unspecific amplification products and of the number of cycles performed, the ratio between the amount of the amplified products exactly reflects the ratio between the amounts of the two RNA species initially present in the reaction, according to the principles of competitive PCR [Wang, 1989] [Gilliland, 1990] [Diviacco, 1992] [Menzo, 1992]. Since the competitor RNA species is added in known amounts, the amount of the unknown target RNA can be easily derived.

The main problem in performing quantitative RT-PCR experiments for RNA is the construction of the competitor RNA. This competitor was obtained according to the procedure outlined in Figure 3. One oligonucleotide complementary to the RNA sequence in close proximity to an intron/exon boundary (in order to avoid subsequently amplification from genomic DNA contaminating the RNA sample) was chosen and utilized for cDNA synthesis with reverse transcriptase (step a). Next, the same oligonucleotide and a novel oligonucleotide with its 3' portion identical to an RNA sequence spaced few hundred nucleotides upstream of the first one, and a 5' unrelated tail containing the recognition sequence for T7 RNA polymerase were used for a first PCR amplification step, resulting in a DNA fragment identical to a portion of the RNA plus a T7 signal at one extremity (step b).

At this point, two novel oligonucleotides (internal primers) were utilized,

one for the sense and one for the antisense strands, corresponding to contiguous sequences at the 3' end and bearing a 20 nt long unrelated sequence at the 5' end (step c). The two unrelated sequences were chosen in order to be complementary to each other. These primers were used in two separate PCR amplification reactions (step d), in each of which one of the external primers was utilized as opposite strand primer. The two PCR products, which contain two contiguous sequences of the original fragment plus the 20 bp unrelated sequence at one extremity, were eluted, mixed together, annealed (step e) and amplified with the external primers, to obtain a DNA fragment corresponding to the original amplification product plus the 20 bp in the middle (step f). RNA can be directly obtained from this fragment by run-off in vitro transcription with T7 RNA polymerase; the amount of the RNA obtained can be exactly evaluated by the inclusion in the transcription reaction of an aliquot of labeled UTP. The RNA obtained by this method can be directly used in competitive RT-PCR experiments, since it is identical to the target sequence to be amplified, except for the 20 nt insertion in its middle. The procedure utilized for the construction of the competitor RNAs is detailed in Material and Methods for the CGD and the ß-actin transcripts.

❸ Quantitation of the gp91 mRNA from CGD patients and normal individuals

Two competitor RNAs were constructed for competitive RT-PCR experiments on clinical samples, as described in the previous section and detailed in Materials and Methods. These competitors allow absolute quantitation of the ß-actin mRNA (an abundant messenger present in almost any cell as a constituent of the cell cytoskeleton) and of the gp91-phox mRNA.

Ten milliliters of blood were drawn from two normal individuals and two affected children (A.G., A.Z.); peripheral mononuclear cells were isolated by density

gradient centrifugation and granulocytes were recovered after red blood cells ipotonic lysis. Aliquots of ~10^7 granulocytes were used for RNA extraction.

Different amounts of competitor RNAs for ß-actin and gp91 (from 10^1 to 10^3 molecules for gp91 and from 10^5 to 10^7 molecules for ß-actin) were added to each sample in two independent quantitation experiments. These competitor species, and the corresponding cellular mRNAs were then submitted to reverse transcription and PCR amplification. By this method, the addition of competitor RNAs before the PCR amplification cycle also allows the control of the efficiency of the retrotranscription step.

The two amplification products for competitor and template were then resolved on a polyacrylamide gel, stained with ethidium bromide and photographed. The equivalence point (i.e. the concentration of competitor which roughly gives rise to an amplification product in the same amount as the cellular mRNA product) was estimated, and a more precise quantitation of the absolute amounts of cellular mRNAs was then obtained by a second competitive RT-PCR experiments with two-fold dilutions of competitor centered around the equivalence point. Figure 4 shows two competitive RT-PCR experiments with two normal individuals for the gp91 gene. A fixed amount of sample RNA (1 µl, roughly corresponding to 50 ng total RNA) were co-amplified with decreasing amounts of competitor RNA (lanes 1 to 5 in panel A and 2 to 6 in panel B). The equivalence point can be detected in lane 4 for panel A (corresponding to $8x10^2$ molecules of competitor) and in lane 4 for panel B (corresponding to $4x10^2$ molecules of competitor). It has to be noted that the presence of unspecific amplification products (see high molecular weight bands in both gels) does not affect amplification, since it interferes equally with both the species.

The results of the quantitation of the absolute amounts of gp91 and ß-actin mRNAs in peripheral granulocytes of two normal individuals and the two CGD

patients are shown in Figure 5. Since the total amount of extracted RNA cannot be estimated (being too small), it is not possible to express the results as absolute number of molecules per total RNA; therefore, this value is expressed as relative to the volume of blood from which the extraction was performed. Since ß-actin is expressed at constant levels in several cell types (including granulocytes), it can be used as a standard for quantitation. It is evident from the results obtained that the gp91 gene is expressed 0.5-1x10^4 times less than the ß-actin gene, a result that further stresses the need of a very sensitive technique for its detection. Surprisingly, no mRNA for gp91 could be detected in both patients, in conditions where the added competitor could be clearly be identifiable after RT-PCR. The ratio between the amount of ß-actin and gp91 mRNAs are reported in the graphic in the lower part of Figure 5. These results clearly indicate that in these affected children the gp91 mRNA molecules are at least two order of magnitude lower than in normal individuals, suggesting that the gp91 gene expression is highly impaired.

❹ Establishment of lymphoblastoid cell lines from CGD patients

Lymphoblastoid cell lines were established for the three patients analyzed in this study. In fact, since the amount of blood that can be drawn from these children is limited, B cell lines offer a very interesting source of unlimited supply of DNA for all the studies aiming at the definition of the molecular defects of these patients.

Two further reasons prompted us to establish these cell lines. First, it has been shown that these immortalized lymphocytes express, although at a very low amount, functional cytocrome b^{558} one their membrane [Volkman, 1984], and, therefore, they can be utilized to study of the functionality of the various subunits of the oxidase by complementation experiments with membrane preparation from other patients or purified proteins; second, they constitute a very useful tool for

monitoring gene expression after transfection of expression vectors for the gene in the development of strategies for gene therapy.

As source of immortalizing virus, the supernatant of the marmoset cell line B95-8 containing high titers of infectious Epstein Barr Virus (EBV) was added to purified mononuclear cells of the patients separated by density gradient centrifugation from blood samples drawn within 24 hours. Cyclosporin was added to the cultures in order to avoid activation of reactive T lymphocytes clones previously sensitized against EBV antigens (the serological reactivity to EBV of the three patients was not known). Clusters of EBV-infected cells started to develop after few days, and increase in the following weeks overgrowing the culture. They were subsequently expanded and frozen as immortalized stocks of patients' derived cells. DNA samples for Southern blot analysis were extracted from these cell lines.

DISCUSSION

The study presented in this thesis concerns some preliminary results about the characterization of the molecular defects in two children affected by chronic granulomatous disease. Furthermore, to this end, a method of wide usefulness has been developed to quantify low amounts of mRNA present in biological tissues by competitive PCR. This constitutes the first step in a long-term study aiming at the development of a protocol for gene therapy in patients with CGD.

Three patients were enrolled in this study; however, samples from one of the three were not immediately available, and therefore he is not included in this preliminary report. All the three children are male, with a long history of recurrent infections of increasing severity, and a diagnosis of X-linked chronic granulomatous disease. Apart from the genetic evidence of transmission of the disease, the results of the NBT (nitroblue of tetrazolium) assay (which tests the proficiency of phagocytes to undergo the oxidative respiratory burst) supported this diagnosis, showing that the granulocytes of the patients are absolutely unable to reduce the dye, while the granulocytes of their mothers show a 50% positivity and those of the fathers are normal. These data are obviously compatible with an X linked transmission form of the genetic defect.

Establishment of lymphoblastoid cell lines

As a first step in the study of these patients, we established lymphoblastoid cell lines by Epstein Barr Virus infection. These cells lines, in fact, can be used as an immortalized source of DNA of these patients, and they were subsequently utilized for DNA extraction for Southern blotting. Furthermore, it was demonstrated [Volkman, 1984] that these cell also express a certain amounts of functional cytocrome at their membrane, and that, after treatment with phorbol myristate acetate (PMA), they exhibit an NADPH-dependent oxidase activity capable of

generating the superoxide anion, similar to, but less efficient than that of activated neutrophils [Cohen-Tanugi, 1991]. As a consequence, membrane preparations from these cells can be used in reconstitution experiments with membrane preparations from other patients (defective in other components of the oxidase system) in order to reconstitute the production of superoxide anion. In addition, since the gene of the gp91 (which is physiologically transcribed exclusively in myeloid cells within the organism) is nevertheless expressed in these transformed lines, these cells can be used as a source of mRNA for cDNA cloning and for all the studies aiming at the definition of point mutations present within the transcript.

Finally, these cell lines can become also a very useful tool to monitor the expression of the normal gp91 gene after transfection and expression from a vector. Obviously, the development of such an assay is the first step to monitor the efficiency of a gene therapy protocol for these patients.

Southern analysis of DNA from the patients

Southern blot analysis was performed with the DNA extracted from the lymphoblastoid cells of the two affected children and of one normal individual after digestion with two different enzymes. The results clearly indicate that there is not any evident difference among the restriction patterns, suggesting that no gross deletions are present in both patients and that, probably, point mutations can explain the genetic defect. This was, indeed, the most probable result we could have expected: in fact it has been described that gross deletions are invariably associated with very complex clinical pictures resulting from deletions of large portions of the X-chromosome [Orkin, 1989], while the two patients studied show only signs and symptoms related to CGD. Furthermore, gross deletions are less frequent than point mutations [Orkin, 1989].

As far as it concerns the presence of typical mutations responsible of the

disease, as in other genetic diseases such as cystic fibrosis, unfortunately a survey of literature revealed that a large number of point mutations have been already described and that they affect almost any portion of the gp91 gene without any preferential localization (for a review see [Smith, 1989]).

Competitive RT-PCR for the detection of low abundance mRNAs

Subsequently, the expression of the gp91 gene was studied. Since this mRNA is expressed at a very low level and exclusively in myeloid cells, we decided to develop a system of detection of small amounts of RNAs by quantitative PCR. It is well established that PCR is the technique of choice for detection of nucleic acids present in low amounts in biological samples, and that its detection limits are close to a single molecule. Quantitation by PCR, however, is problematic, since the final yield of the amplification reactions can be affected by several parameters, some of which can be hardly controlled by the operator, even in the most controlled experimental conditions. Since the final product derives from exponential amplification of the starting template, minor differences in amplification efficiencies (especially in the first cycles) will result in large differences in the overall product yield, especially if the amount of initial template is low. For this reason, a reliable method for quantification is essential. Several authors have observed a linear relationship between input template and amplification product within the exponential range of amplification [Oka, 1990] [Rappolee, 1989] [Singer-Sam, 1990] [Delidow, 1989] [Chelly, 1989] [Arrigo, 1989] [Schnittman, 1989]. This range, however, is strictly dependent on the abundance of the starting material (the more abundant the material, the shorter being the range [Chelly, 1990], and is heavily influenced by differences in sample preparation, machine performance, reaction conditions, and presence of inhibitors. Similar problems must be faced by methods using limiting dilution analysis of the sample [Simmonds, 1990]. For all these

reasons, although semiquantitative data can be obtained readily with dilution curves, quantitative analysis is cumbersome and difficult.

An approach to overcome these tube-to-tube variations has been the co-amplification within the same tube of a reference template, being a single copy cellular gene [Kellog, 1990] [Pang, 1990] [Frye, 1989] [Neubauer, 1990] or an ubiquitously expressed mRNA [Chelly, 1989]. The principle of the technique is that any variable influencing amplification should affect both the reference and the template under study similarly, if the reaction is maintained into its exponential phase. However, even if all the amplification parameters for each primer set are previously empirically determined, nevertheless the nature of the amplified sequences and of the primers have a largely unpredictable influence on the efficiency of amplification.

For all these reasons, the most reliable approaches to quantitative PCR are those based on co-amplification of reference templates that share with the target sequence the same primer sites and the near totality of the amplified sequence, so that the two templates compete for the same primer set and subsequently amplify at the same rate (competitive PCR). The two amplified products can be recognized because of their different lengths [Seibel , 1991] [Ozawa, 1990] [Gilliland, 1990] [Wang, 1989], or for the presence of a mutation in the competitor which creates a novel restriction site [Becker-André, 1989] [Gilliland, 1990] or can be resolved by temperature gradient gel electrophoresis [Henco, 1990].

However, since natural competitor sequences are not often available, the major problem suffered by competitive PCR is the construction of competitors, which can be often a tedious and long work of mutagenesis and cloning. The most relevant characteristics of the methodology we developed is the use of a competitor RNA molecule which shares the same sequences as the target mRNA molecule, except for a 20 nt insertion in its middle, and is directly derived from the

amplification product by an application of the recombinant PCR technology [Higuchi, 1988] [Ho, 1989] without need for cloning. The addiction of competitor RNA molecules to the RNA sample has also another advantage over non-competitive techniques or techniques using DNA competitors, namely the fact that both target and competitor are also equally reverse transcribed in the cDNA synthesis step [Menzo, 1992] [Diviacco, 1992].

Expression of the gp91 gene in peripheral blood granulocytes

Two different competitor RNA molecules were constructed, for the absolute quantitation of the ß-actin and the gp91 transcripts. Since ß-actin is a widely and constantly expressed gene, it can be used as standard for RNA quantitation, and as a control of the quality of the RNA samples tested. The results obtained for the quantitation of the two RNA species in the two affected children and two normal individuals allowed us to conclude that the normal levels of expression in peripheral granulocytes of the gp91 gene are $0.5\text{-}1 \times 10^4$ times less than those of the ß-actin gene (confirming that it is a low-abundance messenger), and that the number of mRNAs for gp91 in granulocytes of the patients is at least two orders of magnitude lower than in healthy donors. It has to be stressed that, since the mRNA for gp91 is under the level of detection even with this very sensitive technique, only the addition of a competitor RNA can guarantee that the RT-PCR reactions were successfully conducted and that the absence of amplification for the template was due to true absence of the template.

Several hypothesis can be formulated to explain the presence of an apparently intact gene and the absence of the corresponding transcript, namely the presence of point mutations in the promoter region altering transcription initiation, or of mutations present in the transcribed region responsible for an altered mRNA stability and/or processing, or, finally, the presence of mutations that create a novel

transcription termination site within the coding region. Sequence analysis of the gp91 coding and promoter regions will allow to discriminate among these possibilities.

Since this method for quantitation of gp91 transcription is suitable for the detection of very low amounts of template RNA, it will also utilized to monitor expression of the cDNA of the gene transfected under the control of a promoter in bone marrow cells of the patients, as a first step for monitoring the efficiency of gene transfer techniques for a gene therapy approach.

Chronic granulomatous disease as a model disease for gene therapy

The current therapeutic strategy in chronic granulomatous disease consists in prophylaxis and treatment of infections. The use of γ-interferon is presently under evaluation: some reports describe an increase of the production of superoxide anion during treatment [Ezekowitz, 1988]. Transfusion of neutrophils was reported to be useful in very severe infections [Fanconi, 1985], but anti-HLA immunoreactivity rapidly develops, which is particularly dangerous if bone marrow transplantation has been planned for the patient.

Also the present experience in bone marrow transplantation is not very encouraging. Only in one patient remission was obtained lasting some time after grafting; the other cases relapsed [Goudemand, 1976]. Finally, serious GVH was described in patients grafted after total body irradiation [Rappeport, 1982].

These therapeutical failures, together with the seriousness of the disease suggest that CGD is an important candidate for gene therapy; in particular, the X-linked form of the disease has some particularly interesting features [Karlsson,1991] for a gene therapy approach. In fact, it is a disease:

- caused by a single gene defect;
- the gene has been cloned;

- the gene is expressed exclusively in cells of the hematopoietic system;
- only 5% of the normal amount of protein is needed to ensure a normal phenotype.

The targets for gene therapy in CGD are the bone marrow stem cells: by transfection of these precursors it should be possible to obtain neutrophils and macrophages able to produce normal levels of superoxide. Furthermore, transfected stem cells should provide continuous production of functional phagocytes, definitely curing the disease, on the contrary of other strategies involving treatment of differentiated, peripherical cells.

MATERIALS AND METHODS

❶ Transformation of B lymphocytes by EBV virus

Culture of the lymphoblastoid starter cell line B95-8 and purification of EBV

PPLO-free cells of the EBV-infected marmoset cell line B95-8 were grown in RPMI 1640 additioned of 50 μg/ml gentamicin, 10% fetal calf serum and 2 mM L-glutamine. When the cells reached a concentration of about 10^6/ml, the EBV-containing supernatant was harvested by centrifuged at low speed (1200 rpm) to remove cells and debris. The supernatant was then passed twice through a 0,45 μM membrane filter (Millex, Millipore) to further remove cells and debris. This virus preparation was kept at 4°C for several days.

Just before use, this supernatant was diluted 1:1 with fresh medium additioned of 50 μg/ml gentamicin, 10% fetal calf serum and 2 mM L-glutamine.

Ficoll separation of PBMC

10 ml of the heparinized blood samples from normal individuals and affected children were processed within 24 hours from withdrawal. They were mixed with 10 ml of RPMI 1640, layered over 10 ml of Ficoll Hypaque (Sigma, S.Louis, MI, USA) and centrifuged for 40 min at 1200 rpm. The peripheral blood mononuclear cells (PBMCs) ring was recovered with a Pasteur pipette, transferred to a new tube and washed three times with RPMI 1640.

Establishment of B lymphoblastoid cultures

Total PBMCs were resuspended in the supernatant of the B95-8 cell line (diluted 1:1 with fresh medium), at a cell concentration of $2x10^6$/ml . One ml of culture was established in sterile 16 well plates, pH was adjustest to 6.8 by CO_2 addition, and the culture was incubated at 37°C. Half of the virus-containing

medium was replaced at the latest 24 hours after starting of the cultures by addition of RPMI 1640 with 20% fetal calf serum, 2 mM L-glutamine, antibiotics and 2 µg /ml (final concentration) of cyclosporin A (Sandimmun,Sandoz). The medium was then refreshed once a week by removing half of the supernatant and replacing it by fresh medium containing 1 µg/ml of cyclosporin A.

❷ DNA extraction from lymphoblastoid cells

Established B lymphoblastoid cells from the patients and from normal individuals were grown in RPMI 1640 additioned of 50 µg/ml gentamicin, 10% fetal calf serum and 2 mM L- glutamine. 10^8-10^9 cells were centrifuged (10 min at 1000 rpm), washed twice with PBS, and the pellet resuspended in 2 ml of lysis buffer B (SDS 1%, EDTA 0.5 M, proteinase K 1 mg/ml). After overnight incubation at 55°C, 1 volume of phenol:chloroform:isoamylic alcohol (25:24:1) was added. The tube was inverted gently and centrifuged 5 min at 3000 rpm at room temperature. The supernatant was then transferred to a new tube, extracted with ether, and dialyzed three times against 2-3 liters of TE (Tris 10 mM, EDTA 1 mM). The amount and the quality of the extracted DNA was analyzed by spectrophotometric analysis.

❸ Southern blot analysis of genomic DNA

Ten µg of DNA extracted from established lymphoblastoid cells of one healthy donor and of two affected individuals were digested with the restriction enzymes *Hind III* and *Eco RI. The* digested DNA was electrophoresed in a 0.8 % agarose gel for 30 h. Southern blotting was performed as described by [Sambrook, 1989]. A *Pst I-Sac* I fragment of plasmid Bluescript-CGD, containing the ~1.6 Kb coding region of the cDNA of gp91-phox cloned into the *Pst I-Xho I* sites of Bluescript KS (Stratagene, La Jolla, CA, USA) was used as probe. The plasmid was a kind gift of Dr. Mary Dinauer. The insert was recovered after digestion by the

low melting agarose technique [Sambrook, 1989] and labeled by the random priming technique with a Promega kit (Madison, WI, USA) according to the manufacturer's instructions.

❹ Separation of PMN from whole blood and RNA extraction

Ten ml of peripheral blood of affected individuals were mixed with 5% dextrane to obtained a 1% dextrane final concentration. After 30 min sedimentation at room temperature, the upper phase (containing serum and white cells) was diluted 1:1 with RPMI 1640 and centrifuged over Ficoll Hypaque gradient for 40 min at 1200 rpm. After separation of the mononucleate cells, 5 ml of EDTA 1 mM were added to the pellet, containing granulocytes and contaminating erytrocytes, in order to destroy erytrocytes. After a further addition of 5 ml of NaCl 1,8% to reconstitute osmolarity, granulocytes were pelleted by a 5 min centrifugation at 1000 rpm. The recovered granulocytes were aliquoted in about 10^7 cell aliquots, and to some aliquots 1 ml of 4 M guanidinium thiocyanate was added for subsequent RNA extraction; the samples were immediately frozen and kept at -80°C. Total RNA from granulocytes was extracted by the guanidinium thiocyanate/acid phenol method described in [Chomczynski, 1987].

❺ Quantitative RT-PCR

Construction of competitor RNA

Competitor RNA fragments were constructed for quantification of the ß-actin mRNA (to be used as internal standard for total RNA quantitation) and of the gp91-phox mRNA from normal individuals and CGD patients by an application of the recombinant PCR methodology [Higuchi, 1988] [Ho, 1989]. The sequence of the oligonucleotides utilized in this work and the amplification conditions are reported in Figure 6.

Oligos CGD4 and BA2 are complementary to the gp91 transcript (exon 13) and the ß-actin transcript (exon 3), respectively. They were utilized as primers for reverse transcription (see below for conditions). The cDNA obtained was amplified with primers CGD4 and CGD3/T7 for gp91 and BA2 and BA1/T7 for ß-actin. Primers CGD3/T7 and BA1/T7 contain a region at the boundary between exon 12 and 13 of the mRNA for gp91(193 nt upstream of primer CGD4) and to exon 2 of ß-actin (196 nucleotides upstream of primer BA2), respectively, at the 3' end (Figure 6, plain typed nucleotides), preceded by a a sequence recognized by T7 RNA polymerase (bold typed nucleotides) and a sequence recognized by the *Bam HI* restriction enzyme (italic typed nucleotides) at the 5' end. The conditions for PCR amplification were the following: 94°C for 1 min denaturation, 54°C for 45 sec annealing and 72°C for 1 min polymerization, repeated 40 cycles.

Two further oligonucleotides were synthesized for the two amplification sets (CGD+3 and CGD+4 for gp91 amplification and BA+1 and BA+2 for ß-actin amplification). These primers have 20 nt at their 3' end identical to contiguous sequences on the upper and lower strands of the amplification products, and 20 nt at their 5' ends complementary each other and unrelated to the amplification products (Figure 6, bold typed nucleotides: this sequence contains three restriction sites for the restriction enzymes *Pst I*, *Bam HI* and *Hinc II*).

Two separate amplifications were carried out for both the gp91 and ß-actin sets of oligonucleotides, with one of the external primers and the internal primer on the opposite strand (primers CGD3/T7 plus primer CGD+4, CGD4 plus CGD+3, BA1/T7 plus BA+2, BA2 plus BA+3). These amplification products, which contain a single overlapping region of 20 bp, were resolved on a 8% polyacrylamide gel, stained with EtdBr, and the corresponding bands touched with the tip of a needle which was subsequently soaked in a single test tube for the two CGD half-amplification products containing 50 µl of distilled water and in another single test

tube for the two ß-actin half-amplification products. After 2 min at room temperature, 5 µl of the elution was included in a standard 100 µl PCR amplification mixture containing only the two outside primers (CGD3/T7 plus CGD4 and BA1/T7 plus BA2, respectively). In order to allow the formation of an heteroduplex product annealed at the complementary sequence with 5' protruding ends (the only product which could be further extended and amplified), the reaction was denatured at 94°C for 1 min, and then the temperature was slowly lowered to 50°C within 10 min. After further 2 min at 50°C, the reaction was incubated for 5 min at 72°C for extension of the annealed products and then amplified using the following PCR cycle profile: the first 5 cycles: 94°C for 1 min, 37°C for 30 sec. and 72°C for 30 sec.; cycles 6 to 10: 94°C for 1 min, 42°C for 30 sec. and 72°C for 30 sec.; cycles 11 to 30: 94°C for 1 min, 55°C for 30 sec. and 72°C for 30 sec.

The amplification products obtained by this recombinant PCR technique has exactly the same sequence as the cDNA of the two transcripts except for the 20 bp insertion in the middle and the addition of a tail with the recognition sequence of T7 RNA polymerase.

RNA competitors were directly obtained from these amplification products (without need for cloning) by in vitro transcription with T7 RNA polymerase using an in vitro kit from Promega (Madison, WI, USA) according to the manufacturer's instructions. Forty ng of DNA amplification product was used as template for the reaction.

Quantification of competitor RNA

Quantification of competitive templates was directly obtained by evaluating the amount of incorporated [^{32}P]UTP in the in vitro transcription reaction as follows.

Two microliters of [^{32}P]UTP (Amersham, U.K.; 3000 Ci/mmole; 10 mCi/ml) were included in the reaction, corresponding to 2.07x10^{7} cpm, as experimentally

evaluated by Cerenkov counting in a beta-counter.

After the transcription reaction, template DNA was removed either by DNase I digestion or by resolution of newly synthesized RNA by denaturing gel electrophoresis [Sambrook, 1989] and elution from the gel.

An aliquot of the purified competitor RNA preparation was counted and its concentration was evaluated from the final specific activity of the labeled UTP and the number of nucleotides incorporated/molecule.

Quantitation of gp91 and ß-actin mRNAs in clinical samples

RNA extracted from granulocytes of normal individuals and CGD patients was submitted to competitive RT-PCR experiments with the ß-actin and the gp91 competitor RNAs. Four microliters of competitor RNA, containing different absolute amounts of molecules (from 10^1 to 10^3 molecules for gp91 and from 10^5 to 10^7 molecules for ß-actin) were mixed to 1 µl of the RNA samples (corresponding to 1/30 of the total RNA extracted from 10^7 granulocytes and reverse transcribed in 50 mM Tris, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 20 U RNasin (Promega, Madison, WI), 1 mM each dNTP, 20 pmoles of primer and 200 U MuLV reverse transcriptase (BRL, Gaithersburg, MD) (final reaction volume: 20 µl). RNA and primers were annealed in reaction buffer and RNasin for 10 minutes at 65° C and then cooled on ice before addition of nucleotides and enzyme. The reaction mixture was overlaid with 60 µl of mineral oil (Sigma, St. Louis, MO) and allowed to proceed for 45 minutes at 42° C. The reaction was stopped by heating at 95° C for 5 minutes.

Eighty microliters of a solution containing 250 µM each dNTP, 10 pmoles of the primer used for cDNA synthesis, 30 pmoles of the other primer, 2.5 U of Taq polymerase (Amplitaq, Perkin Elmer Cetus, Norwalk, CT), 50 mM KCl, 10 mM Tris, pH 8,3, 1.5 mM $MgCl_2$, 0.01% gelatin were directly added to the 20 µl used for

cDNA synthesis. Reaction mixtures (100 µl) were subjected to 40 cycles of amplification in a programmable thermal cycler (Perkin Elmer Cetus, Norwalk, CT) using the profiles reported in Figure 6.

Amplification products were resolved on a 8% polyacrylamide gel, stained with ethidium bromide and photographed.

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