ABSTRACTS

The 2nd Workshop on PCR in Medicine, 8. 11. 2000 in Brno, the MEFA Trade Fair Scientific Programme

organised by
Sigma-Aldrich and BVV vompanies
under the auspices of
Professor MUDr. J. Žaloudík, CSc.
Head of the University Cancer Centre in Brno

T. Freiberger[1], *J. Mayer*[2] ([1]Department of Immunogenetics, Research Institute of Child Health, Brno; [2]Department of Internal Medicine – Haemato-Oncology, University Hospital, Faculty of Medicine, Masaryk University, Brno): **Detection of chimerism in patients after allogeneic peripheral blood stem cell transplantation with non-myeloablative conditioning**

Allogeneic bone marrow transplantation (BMT) is a promising therapeutical tool for many malignant and non-malignant diseases. Complete donor haematopoiesis or donor chimerism in the organism of a recipient are markers indicating good transplant engraftment. Recently, there has been some proof of mixed chimerism (MC), e.g. survival of the recipient's cells, being compatible with long periods without disease relapse. On the other hand, some authors consider mixed chimerism to be a risk factor for relapse.

Clinical interpretation of MC is influenced by several factors, one of which is the sensitivity of the method used for chimerism detection. From this point of view, a breakthrough point was the introduction of molecular biology methods for analysis of genetic material (DNA) of both donors and recipients. However, the DNA detection methods themselves have various degrees of sensitivity. Very low levels of MC (< 1% of recipient's nuclear cells) are probably not related to a recurrence of disease. Another factor is the dynamics of recipient's cell percentage after transplantation. An increasing number of the recipient's cells is supposed to carry a higher risk of relapse; however, stable or transient MC may not be related to relapse. Moreover, transient mixed chimerism may protect the host from severe acute GVHD. A very important fact is that the changes detected at the DNA level precede the changes visible in the patient's blood counts and clinical manifestations of the disease. This is of major importance in timing a therapeutical intervention. There is also evidence suggesting that mixed

chimerism is present only in some cell lines, and its detection may be very important for determinig the risk of relapse. The degree of MC may also be influenced by various protocols used to prepare patients for BMT, by various ways of transplantation itself (non-manipulated or T-lymphocyte depleted bone marrow) or by various post-transplantation therapeutical approaches.

Non-myeloablative conditioning is a very hopeful, alternative approach. The dynamics of chimerism after non-myeloablative transplantation has not been sufficiently studied. The authors present their first experience with the detection of chimeric status, using methods of DNA analysis, in patients transplanted after the non-myeloablative regimen.

J. Šmardová, A. Nemajerová, Š. Pavlová (Department of Cellular and Molecular Oncology, Masaryk Memorial Cancer Institute, Brno): **Functional analysis of separated alleles in yeast: analysis of the tumour suppressor p53**

The p53 tumour suppressor gene encodes a sequence-specific DNA-binding protein. The wild-type p53 protein acts as a "guardian of the genome". In response to DNA damage, p53 triggers cell cycle arrest in the late G_1 phase to allow for DNA repair prior to its replication, or it may induce apoptosis. The loss of p53 function permits propagation of genetically damaged cells, which explains why p53 inactivation is a common step in cancer development.

FASAY (functional analysis of separated alleles in yeast) is a functional analysis testing the transactivation capability of p53 protein. The central part of the p53 gene derived from mRNA of tumor (peripheral blood, tissue culture) cells by RT-PCR is introduced into an Ade^- yeast strain carrying a reporter with a p53-binding site RGC (ribosomal gene cluster) upstream of the ADE2 gene. Yeast cells grown in agar plates, which contain a low level of adenine, form either bigger white colonies, if the reporter is activated by p53, or smaller red colonies if the reporter is not activated (the colonies are smaller because their growth is limited by the low amount of adenine in the medium and their red colour is caused by accumulation of a reddish product of adenine metabolism). When using FASAY for p53 status analysis, only a background of up to 10% of red colonies is expected for the cells that have both functional alleles of the p53 gene. Samples providing more than 10% of red colonies are considered positive for a p53 mutation. FASAY is very sensitive to the quality of mRNA and also to the quality of DNA polymerase. It can be used to measure DNA polymerase fidelity.

FASAY was used in several clinical studies for detection of somatic as well as germ-line p53 mutations. In our laboratory we used FASAY for p53 status analysis in breast and cervix cancer samples and in acute myeloid leukaemia blood samples.

This work was supported by grant no. NC/6395–3 from the Internal Grant Agency of the Ministry of Health of the Czech Republic.

D. Škapová[1,2]*, D. Dvořáková*[1]*, Z. Křístková*[1]*, J. Sučiková*[1] ([1]Department of Internal Medicine – Haemato-Oncology, University Hospital Bohunice, Masaryk University, Brno, [2]Department of Biology, Faculty of Medicine, Masaryk University, Brno): **The use of PCR in the early detection of pathogens which complicate the treatment of patients with haematological malignancies**

Haematopoietic stem cell transplantation (HSCT) is a method of treatment in several haematopoietic and lymphoid disorders. The therapies used to prevent rejection suppress the immune system and, as a result, the transplant recipient is often at a high risk of infection. The pace of gradual reconstitution of the immune system after transplantation is a factor determining the type of infectious complication which may be caused by bacterial, viral, fungal or parasitic pathogens. It is known that infectious complications remain a major cause of morbidity and mortality after HSCT.

Preventive, pre-emptive and therapeutic approaches have been developed which facilitate better control of infections. However, they require very specific and sensitive methods of pathogen detection.

Polymerase chain reaction (PCR) is a technique that can detect small amounts of pathogen nucleid acids (DNA, RNA) in the sample. This method utilises repeated cycles of oligonucleotide-direct DNA synthesis in order to perform *in vitro* replication of target nucleic acid sequences. Several modifications of the basic PCR procedure have been described.

We demonstrated the diagnostic value of PCR assays (non-nested, nested- and semi-nested-PCR, RT-PCR) for the detection of infectious agents in clinical specimens collected from patients after peripheral blood stem cell transplantation (PBSCT). With the use of these assays, human cytomegalovirus (HCMV), HCMV-resistant strains, human herpesvirus 6 (HHV-6) and *Pneumocystis carinii* were identified.

R. Vodička, R. Vrtěl, J. Zapletalová, D. Konvalinka, K. Adamová, A. Šantavá, E. Krejčiříková, J. Šantavá (Department of Medical Genetics and Foetal Medicine, University Hospital of Palacky University, Olomouc): **Molecular and molecular cytogenetic analysis of Y-specific DNA sequences in patients with Turner's syndrome**

Turner's syndrome (TS) is associated with chromosome X monosomy. More than 95% of foetuses with TS are spontaneously aborted. The frequency of TS in the female population is about 1 in 2500. TS is mostly caused by non-disjunction during oogenesis or by retardation of chromosome Y during meiosis. Most of the TS individuals are cytogeneticaly 45,X (50 %), the remaining cases have different types of chromosomal mosaicism or structural abnormalities of chromosome X and also Y.

The presence of DNA sequences derived from chromosome Y appears to be a causal factor in the development of gonadoblastoma in TS patients.The risk of gonadoblastoma in the case of Y-positivity is reported to be about 25 %. The frequency of Y-positive TS patients varies with different authors. Our group of TS patients was examined for anomalies in regions DYZ3 and SRY completely, and in regions PABX/Y and amelogenin partially. In the positive cases we tried to specify the Y chromosome regions, using Y-centromeric and Y-painting probes.

The results of this study can be used as a contribution to the prevention of gonadoblastoma development and clarification of the molecular basis of neoplastic transformation.

D. Dvořáková, J. Zemánková, J. Sučiková, J. Mayer (Department of Internal Medicine – Haemato-Oncology, University Hospital Bohunice, Faculty of Medicine, Masaryk University, Brno): **Detection of minimal residual disease in patients with chronic myeloid leukemia after peripheral blood stem cell transplantation**

Chronic myeloid leukemia (CML) is a clonal, malignant, myeloproliferative disorder of the haematopoietic stem cells. The diagnosis of CML is usually based on detection of the Philadelphia (Ph) chromosome that results from a reciprocal translocation t(9;22).

The molecular consequence of t(9;22) translocation is a product of the fusion bcr/abl gene. This chimeric mRNA transcript represents a leukemia-specific sequence that can be used to identify residual leukemia after haematopoietic stem cell transplantation.

Allogeneic haematopoietic stem cell transplantation provides the most important treatment for CML patients but their relapse is still the major cause of treatment failure. Thus, in CML patients, an evaluation of minimal residual disease aimed at early detection of relapse has relevant therapeutic implications.

We used a highly sensitive, quantitative reverse-transcriptase (RT-PCR) assay, with natural competitors capable of detecting a single bcr/abl, mRNA-expressing cell amongst 10^6 white blood cells, for monitoring the dynamics of the BCR/ABL clone. The test was performed at regular intervals after allogeneic peripheral blood stem cell transplantation (PBSCT). Our protocol of quantitative RT-PCR assay contributes to the characterisation of CML evolution after transplantation and is applicable for early identification of patients at the highest risk of relapse. We conclude that this technique might permit therapeutic intervention at earlier stages of the disease.

J. Drábek, E. Weigl (Department of Immunology, Teaching Hospital, Faculty of Medicine, Palacký University, Olomouc): **Alternatives of PCR for nucleic acid amplification**

The polymerase chain reaction (PCR) is now the most preferred method for nucleic acid amplification because of its universality, specificity, and sensitivity. Although generally neglected, there are several other methods which could be more suitable for certain specific applications.

In this review, the following techniques are compared with PCR: target amplification techniques, i.e., transcription-based amplification, transcription-mediated amplification, nucleic acid sequence- based amplification (also known as self-sustained sequence replication), Q b replication, strand displacement amplification, ligase chain reaction, and boomerang DNA amplification; probe amplification techniques, i.e., cycling probe technology and rolling circle amplification, are also mentioned..

M. Vojtíšková[1]*, M. Falk*[1]*, J. Vytopil*[2]*, G. Enzmann*[3]*, U. Froster*[3]*, R. Gaillyová*[1] ([1]Department of Human Genetics, [2]Department of Neurology, University Hospital Bohunice, Brno, [3]Department of Human Genetics, University, Leipzig): **PCR methods for detection of large trinucleotide repeat expansions**

Trinucleotide repeats have attracted wide attention since their expansion was found to cause a growing number of human neurological disorders including myotonic dystrophy, Huntington®s disease, spinocerebellar ataxia, etc. The molecular diagnosis of these diseases is based on the PCR method which allows us to determine pathological alleles approximately up to 100 trinucleotide repeats and, in the case of myotonic dystrophy (DM), the CTG repeat can expand to give alleles lager than 5 kb. DM is an autosomal-dominant neuromuscular disease with an incidence of 1 in 8000 individuals, thus representing one of the most common forms of adult-onset muscular dystrophy.

The gene initially considered to be the only one responsible for DM is located on chromosome 19q13.3 and it codes for a protein with serine-threonine kinase activity (DMPK). The mutation that gives rise to DM consists of an unstable CTG repeat expansion in the 3'-untranslated region of the DMPK gene. The CTG repeat number ranges from 5 to 30 in normal subjects and expansions up to several thousands of CTG repeats show a positive correlation with the severity of the disease and with its increase in successive generations of DM patients. The exact length of long, pathological alleles is commonly determined by Southern blot analysis, as recommended by the Clinical Molecular Genetics Society. To reduce the time for obtaining final results of DNA analysis in DM patients and to determine correlation between the degree of CTG repeat amplification and the

clinical severity of individual symptoms, we introduced a fast and efficient protocol with the use of PCR methods: a) triplet-primed PCR (TP PCR) and b) XL system for long PCR targets with partial substitution of 7–deaza-dGTP (XL PCR).

Using this PCR protocol, we assessed the allelic frequency of CTG repeats in a group of DM patients in comparison with a control group. In the DM patients, the amplified alleles ranged from a minimum of 100 to more (unlimited) CTG repeats and the 5 CTG repeat allele was present only in 5%, which was much lower than in the control group. Clinically and histologically (muscle biopsy) confirmed subjects with alleles expanded to more than 100 CTG repeats were correctly identified by both TP and XL PCRs, and the exact lengths of very long, pathological alleles were confirmed by Southern blot analysis

I. Šubrt,[1] *I. Reitmaierová*[1]*, D. Sedláček*[2] ([1]Department of Medical Genetics, [2]Department of Infectious Diseases, Faculty Hospital, Medical Faculty, Charles University, Pilsen): **CCR5 32-bp assessment in HIV-positive patients in Western Bohemia**

The chemokine receptor CCR5 is an important surface co-receptor structure necessary for binding HIV-1 to target macrophage cell lines that serve as reservoirs for HIV. The CCR5 protein is encoded by the CCR5 gene. It has been reported previously that subjects with a mutated CCR5 gene are HIV-1 resistant due to CCR5 non-expression. A 32-bp deletion can be found in either the homozygous (1–3%) or the heterozygous (5–18%) form in persons of Caucasian origin.

In our study we analysed genomic DNA samples extracted from buccal cells of 12 HIV-positive patients registered at the AIDS Centre of the Faculty Hospital in Pilsen. We used a new, previously unpublished, primer pair flanking 32-bp deletion region of the CCR5 gene. Subsequent detection of wild-type and mutated allelic variants of PCR amplification products was performed on high resolution agarose gels.

The results showed that our approach is suitable for CCR5 32-bp deletion assessment in both HIV-positive patients and the healthy population.

K. Kaňková[1]*, J. Záhejský*[2]*, I. Márová*[2]*, J. Mužík*[1]*, V. Kuhrová*[1]*, V. Znojil*[1]*, M. Beránek*[1] ([1]Department of Pathological Physiology and [2]Department of Dermatology, Faculty of Medicine, Masaryk University, Brno): **Polymorphisms in the RAGE gene influence susceptibility to diabetes-associated dermatoses in NIDDM subjects**

The formation of advanced glycation end-products (AGEs) plays an important role in the development of late complications in diabetes. AGEs bind to the

receptor of advanced glycation end-products (RAGE). This AGE-RAGE interaction results in generation of intracellular oxidative stress. Genetic polymorphism in the complete sequence of the RAGE gene was analysed. Possible associations with diabetes-associated dermatoses (DAD) and the impact of newly described polymorphisms on the glycation-antioxidative status in NIDDM subjects were investigated.

A part of the RAGE gene spanning a region from -4 to 3334 bp was analysed by means of PCR, with subsequent heteroduplex and SSCP analyses. Allele frequencies and genotype combinations of novel, common polymorphisms were determined in an association study comprising four groups of subjects (n=535). In our NIDDM subjects, the mean duration of diabetes was 6.8 6.3 years. The presence of late diabetic complications in particular localisations was expressed as an index (I_{compl}). The levels of HbA_{1c}, glycated stratum corneum proteins (Amadori and AGE-keratin), total carotenoids, alpha- and beta-carotenes, gamma-tocopherol, lutein, lycopene and alpha-tocopherol were measured in each subject.

Fifteen novel polymorphisms (R77C, G82S, V89V, 718G/T, 1704G/T, 1727A1728ins, H305Q, S307C, 2117A/G, 2184A/G, 2245G/A, 2249A/G, 2741G/A and 3089ACdel) were identified. A significant association with dermatoses, irrespective of NIDDM, was found for mutation in exon 82S (P_{corr}0.05) and a marginally significant association was shown for intron variant 1704T (P_{corr}0.05). Individual genotype combinations of G82S, 1704G/T and 2184A/G were significantly associated with the presence of dermatoses (P0.01). Glycation variables did not reveal any significant associations with any of the polymorphisms analysed. However, significant differences between the genotypes of intron polymorphisms and the levels of total carotenoids, lutein and alpha-tocopherol ($P<0.05$, Kruskal-Wallis ANOVA) were found. I_{compl} significantly correlated with the plasma levels of all antioxidants (all $P<0.01$, Spearman correlation coefficients), while no correlation of I_{compl} with glycation variables was observed.

Polymorphisms G82S, 1704G/T and 2184A/G were shown to influence susceptibility to dermatoses with no relation to diabetes itself. Several polymorphisms in the RAGE gene significantly influenced the antioxidant status in NIDDM subjects. The extent of diabetic vascular disease was shown to be related to the plasma levels of antioxidants.

A. Vašků, P. Beneš (Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Brno): **PCR detection of candidate gene polymorphisms in essential hypertension and coronary artery disease**

Common cardiovascular diseases, i.e., essential hypertension (EH) and coronary artery disease (CAD) are believed to have a multigenic background.

This, in interaction with environmental conditions, is the most important factor in the development of disease. Frequent polymorphisms in different candidate gene sets are supposed to contribute to the manifestation of these diseases.

Seven polymorphisms in the genes coding for angiotensin I-converting enzyme (ACE), angiotensinogen (ATG), endothelin-1 (ET-3) and TNF were detected in three groups of subjects designated A, B and C (n=398; A, patients with normotension; B, patients with hypertension, both confirmed by ambulatory blood pressure monitoring; C, treated hypertensives). No significant differences were found when single gene allelic frequency was investigated. However, more complicated relationships with to hypertension were revealed when allelic associations of every two polymorphisms detected were considered. Several highly significant associations between genotypes and EH were found when risk factors such as alcohol consumption, smoking and body weight were taken into account. The association of DD (I/D polymorphism in ACE) and TT (T174M in ATG) genotypes with EH increased with high alcohol consumption (P=0.005). The significant gene interaction of M235T ATG and TNFβ polymorphisms with alcohol consumption was found in group B subjects who had the TTB1B1 genotype (P=0.000001). Other less significant associations were found for smoking, salt consumption and body weight.

Seven candidate gene polymorphisms (ApoE, ApoB, MTHFR, PAI-1, LRP, RAP and TNFβ) were associated with coronary artery disease (657 patients with CAD and 537 healthy controls) but no significant association was found when the allelic frequency for single polymorphisms was compared between the healthy and ill subjects. Risk factors, such as age, sex, smoking status, body weight, hypertension, diabetes mellitus and hyperlipidemia, contributed to more significant differences between healthy and ill individuals in Apo E polymorphism (sex, P0.05), in Apo B (diabetes, P0.05 smoking state, P0.05 body weight, P0.05 age and sex, P=0.003), in MTHFR (without hypertension, P 0.05) and in TNFβ (without diabetes mellitus, P0.05). The highest difference in ApoB was found when non-diabetic, ischaemic patients were compared with the control subjects, smokers with BMI25 kg/m (P 0.01). It can be expected that even more complicated (interactive) relationships between gene polymorphisms and phenotype characteristics will be revealed in the future.

M. Petřek (HLA Laboratory, Department of Immunology, Palacky University and University Hospital, Olomouc): **Receptor gene expression in chemokines and polymorphism identification: applications to respiratory and forensic medicine**

With regard to chemoattractant properties of chemokines, these low molecular weight cytokines are prime candidates for mediators of leukocyte recruitment to

the sites of inflammation. Confirming the role of chemokines and their receptors in the pathophysiology of inflammatory disease has important implications for the development of novel, anti-inflammatory therapies aimed at blocking chemokine-induced cell migration.

The promigratory function of chemokines has, therefore, been investigated in a wide range of clinical studies which compared chemokine mRNA and protein expression in healthy subjects and in patients with inflammatory conditions including chronic lung diseases such as sarcoidosis or pulmonary fibrosis.

Our own studies were directed at the assessment of mRNA expression for CC & C chemokines in the lower respiratory tract cells. Utilising the RT-PCR-SSP methodology (PCR with sequence specific primers preceded by reverse transcription), we obtained mRNA expression data suggesting the importance of CC & C chemokines (namely RANTES, CCL5 and lymphotactin, CL1) for lymphocyte recruitment to the lungs of patients with sarcoidosis. Our current work aims at differentiation between pathological and physiological chemokine expressions and at a definition of clinical significance of chemokine expression in disease follow-up.

CC chemokines exert their function via specific receptors (CCRs) located in a gene cluster on chromosome 3p21.3 (receptors CCR1, -2, -3 & -5). The genes for CCR2 and CCR5, which bind chemokine ligands implicated in the pathogenesis of sarcoidosis, are polymorphic. When investigating the gene and phenotype frequencies of CCR2 and CCR5 alleles after PCR-SSP genotyping, we observed that the CCR2-64I allele protected from sarcoidosis (odds ratio, OR = 0.5). The CCR532 allele, on the contrary, conferred disease susceptibility (OR 1.9, p=0.02) and, importantly, the patients with this allele were three times more prone to develop a more advanced disease requiring treatment·.

Our laboratory currently investigates genetic susceptibility to sarcoidosis and pulmonary fibrosis by screening a number of biologically relevant, polymorphic genes located on distinct chromosomes. Our study of the genes predisposing to lung disease has another, interesting application in the development of a novel, PCR-based system which may also be utilised in forensic medicine, for example, in parentage testing.

This work, initially funded by the European Community and British Society for Immunology, was also supported by the Czech Government (IGA 3768–3, MŠMT 151100002, GA ČR 310/99/1676).

Sestavil: *R. Janisch*