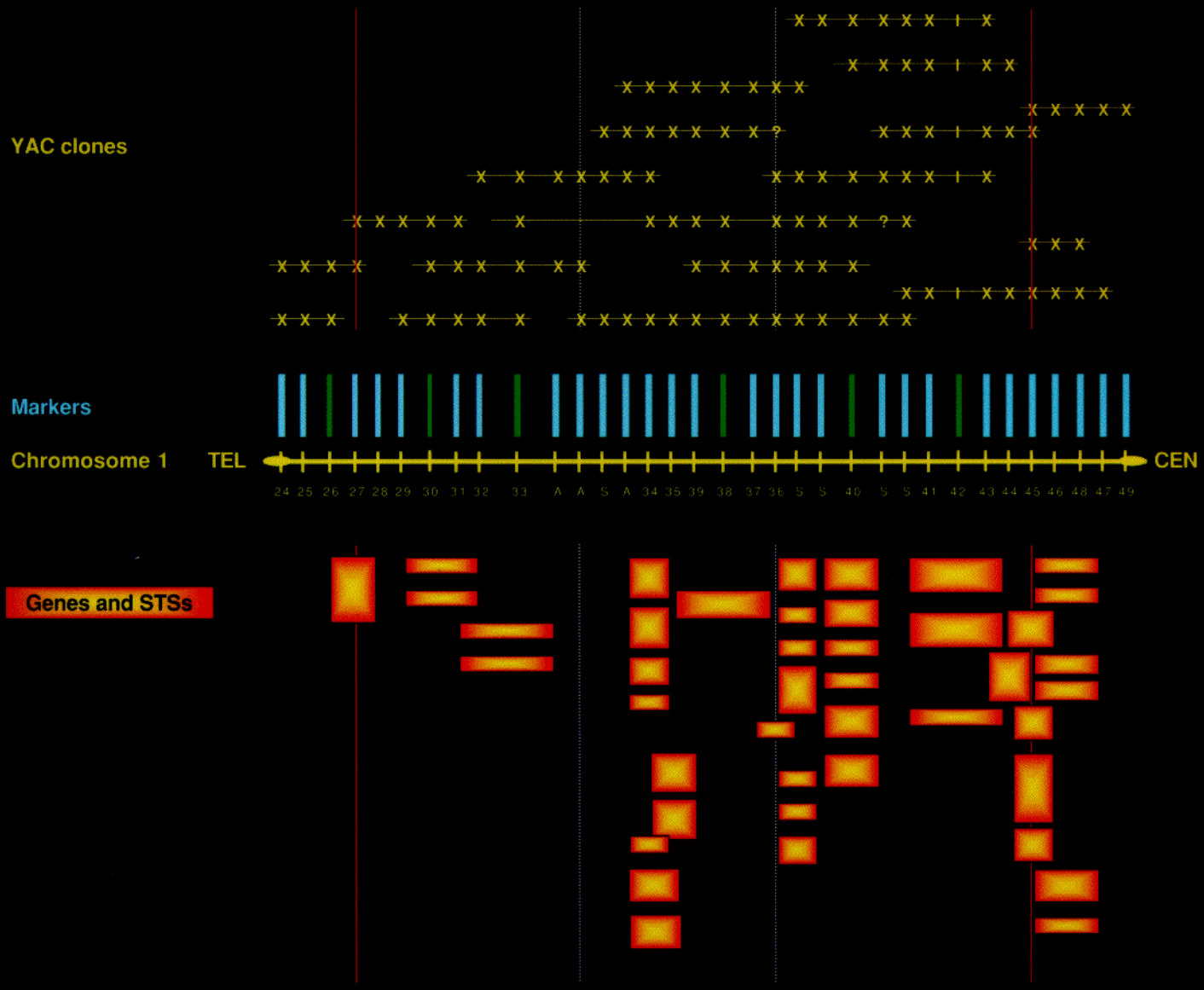
ICP
Christian de Duve Institute
of Cellular Pathology

Scientific report

2000



PHYSICAL MAP OF THE LOCUS FOR GLOMUVENOUS MALFORMATION
ON HUMAN CHROMOSOME 1p21-p22

Front cover :

Physical map of the VMGLOM locus on human chromosome 1p21-p22 (see page 22).
This physical map was created by the group of Prof. M. Vikkula (Human Molecular Genetics), and was used for the successful positional cloning of the gene causing an inherited form of a vascular anomaly, cutaneous glomuvenous malformation. This schematic representation of the physical map is devided into three parts: top (in yellow), middle (in blue and green) and bottom (in orange). Yeast artificial chromosomes (YACs) that were identified to cover the region are marked on top (yellow lines). The positions of the genetic markers that were used (sequence tagged sites, STS) are marked in blue, or in green, in case it also represents a gene (middle part). The orange boxes (bottom part) represent areas of localisation of the newly mapped STSs (markers or genes). Each marker that is present on a given YAC is marked by a small x in the top part (yellow).
The group of Prof. Vikkula defined the *VMGLOM* locus originally using linkage analysis (vertical red lines). They redefined and narrowed the locus using linkage disequilibrium (vertical white lines). By positional cloning, the causative *VMGLOM* gene was ultimately identified in the region defined by the white lines.

ICP A.I.B.S.

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Avenue Hippocrate 75
Boîte 75.50
B-1200 BRUXELLES

Tel. : (32 2) 764.75.50
Telefax : (32 2) 764.75.73
E-mail : administration@icp.ucl.ac.be

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Acknowledgments

The members of the Development and Expansion Council as well as many friends of the ICP have continued to help and support our research efforts, under the generous leadership of their President, Baron PETERBROECK.

The appeal for sponsoring postdoctoral fellowships was widely followed. In 2000 the ICP has been able to allocate the following fellowships, entirely supported by our donors :

the "Haas-Teichen" fellowship to Simone MÜLLER

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Our gratitude goes to all the generous donors.

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The investigators of the ICP are supported financially by the "Université catholique de Louvain", the Belgian State, the Regions, various national, foreign and international foundations and organizations, industries and private philanthropy.A general debt of gratitude is due to the following organizations, which have offered substantial support to the research programme at the ICP in 2000.

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INTRODUCTION

The ICP

The ICP is an international biomedical research institute with a twin objective: to contribute to the advancement of knowledge and methods in the field of fundamental biology and to apply this knowledge to further the health and well-being of all.

Part of the complex of the Faculty of Medicine at the Catholic University of Louvain, the ICP also houses the Belgian branch of the Ludwig Institute for Cancer Research.

The ICP is based on three principles : 1) priority to basic research and to the freedom of the investigators; 2) obligation to help in developing beneficial applications of discoveries whenever the opportunity arises; 3) multidisciplinary collaboration within a critical mass of competences, instruments and techniques.

By giving priority to basic research, the ICP remains faithful to a time-honored tradition of proven value. It is indeed known that a large number of medical advances stem from investigations undertaken without any defined practical objective and aiming mainly at promoting the understanding of the living world. But the ICP's basic investigators differ from their predecessors by a much stronger commitment to applied medical research. Most of them carry out investigations in which the acquisition and the application of new knowledge are intimately linked.

Also characteristic of the ICP are the many collaborations investigators maintain with each other and with other groups, in Belgium and abroad. Such an association is a necessity. Modern biomedical research increasingly demands the participation of different disciplines, each calling on a multiplicity of specialized knowledge and techniques. Hence the need for a critical mass, which only a centre of sufficient size can bring together.

In spite of its relatively small staff, the ICP pursues investigations in a great variety of fields. This diversity, which stems from the principles that have guided the creation of the institute and, notably, from the freedom enjoyed by its investigators, nevertheless does not lead to excessive dispersion of efforts. On the contrary, it generates particularly fruitful exchanges that are made possible, beyond the differences among subjects, by the common concepts and methods characteristic of modern biology. The same tools are used and the same language is spoken, whether one looks at a virus, a microbe, a parasitic protist, a plant, an animal or a human being. Following are the research groups at work in 1999.

Metabolic investigations.

This unit tries to analyse the molecular mechanisms, such as allosteric modifications, phosphorylation of protein and protein-protein interactions, involved in the control of a metabolic pathway by hormones, substrates, drugs and other active agents. The group is also interested in "inborn errors of metabolism", the name given to hereditary diseases due to enzymatic deficiencies resulting from genetic modifications. The unit includes two main research groups.
1) A group, directed by Emile Van Schaftingen, studies the regulation of carbohydrate metabolism, with special emphasis on glucokinase, an enzyme that plays a critical role in maintaining the blood-sugar level (perturbed, among others, in diabetes) and that is controlled by a regulatory protein discovered by members of the group.
2) The other group, directed by Georges Van den Berghe, investigates the deficiency of adenylosuccinate lyase and purine compounds with therapeutic potential in cancer, mainly 2-chloro-2'-deoxyadenosine.

Human molecular genetics.

This unit, directed by Miikka Vikkula, uses molecular genetic approaches to identify genes and mutations causing inherited human disorders. The group is especially interested in the identification of the molecular mechanisms leading to vascular anomalies, cardiopathies, cleft lip and palate, and certain neurological tumors. During the year 2000, these studies have lead to the discovery of causative genes for three hereditary vascular disorders: congenital lymphedema, cutaneous capillary-venous malformation, and glomuvenous malformation. Future studies are aimed at characterizing the role of the encoded proteins in blood vessel development.

Hormones and metabolism.

Jointly directed by Guy Rousseau and Louis Hue, this unit studies the signaling mechanisms of hormones and the control they exert on gene expression. Three themes are under investigation.
1) The regulation of gene transcription by hormones and by tissue-specific and developmental factors, including hepatocyte nuclear factor-6 which was discovered in this laboratory.
2) The regulation of carbohydrate and lipid metabolism by hormones and by ischemia, neoplastic transformation and cell volume.
3) The analysis of the relationship between the structure of an enzyme or protein and its biological function, notably after modification of this structure under the influence of hormones. Their main model is 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase whose mRNAs and genes were originally cloned by this group.

Cell biology and extracellular matrix.

This unit studies cells and tissues in the perspective of their compartmentalisation and communication between intracellular and extracellular compartments. Accordingly, the unit has an established experience in molecular tracking by analytical subcellular fractionation combined with immunocytochemistry, *in situ* hybridization, confocal and electron microscopy, and has gained expertise in co-culture systems and defined proteinase assays. The investigations of the unit are focused on three major topics : (1) endocytosis of exogenous and endogenous (plasma membrane) constituents (P.J. Courtoy); (2) regulation by endocytosis of thyroid hormone production (M.F. van den Hove); and (3) mechanisms and control of extracellular matrix degradation with special emphasis on the human endometrium as a model system (Y. Eeckhout, P.J. Courtoy, E. Marbaix).

Connective tissues.

The research activities of D. Manicourt and his co-workers are centered on the physiopathological events leading to the degradation of connective tissue matrices, particularly those of bone and cartilage.

Infectious pathogeny.

This unit includes two research groups :
1) The group of Guy Cornelis studies the virulence factors of *Yersinia enterocolitica*, a common human pathogen frequently isolated from pork meat. The system *Y. enterocolitica* uses to survive in its host became the archetype of a new virulence mechanism shared by several other pathogenic bacteria, including plant pathogens. While throwing light on molecular communications between the invasive bacterium and the cells of the immune system, these investigations also provide illuminating information on the secretion of proteins by bacteria.
2) The group of Thomas Michiels studies Theiler's virus, a picornavirus responsible for a persistent infection of the central nervous system of the mouse, reminiscent in certain respects of human multiple sclerosis.

Tropical diseases.

Directed by Fred Opperdoes with the help of Paul Michels, this unit investigates parasitic protozoa of the family of Trypanosomatidae, which cause diseases such as sleeping sickness (which prevails in Africa), Chagas disease (widespread in Latin America) and leishmaniases (endemic in many tropical regions).
The aim of the unit is to exploit cellular and metabolic differences between the parasite and the human host in order to develop more efficient treatments against these diseases. The major research topic in this respect is the unique compartmentation of the glycolytic enzymes inside glycosomes, i.e. the peroxisome-like organelles typical of the

Trypanosomatidae. To this end, it uses all the resources of biochemistry, protein biophysics, molecular biology, and molecular modelling.

Immune defences.

Three groups are investigating the mechanisms by which the organism defends itself against invasion by either infectious agents or cancerous cells.
1) Jean-Pierre Vaerman and co-workers are specially concerned with the defence of digestive and respiratory mucosae mediated by a special kind of immunoglobulin, IgA, discovered by the founder of the group, the late Joseph-Felix Heremans.
2) Jean-Paul Coutelier and his co-workers study the profound alterations of the immune system triggered by some viruses, with a special interest to autoimmune disorders.
3) Pierre Coulie and his co-workers, in close collaboration with the Ludwig Institute, work on the anti-tumoral immune mechanisms. This unit is particularly interested in monitoring the specific immune responses developed by patients who are treated by injections of various tumoral antigens.

The Ludwig Institute

Founded in 1971 by the American financier philanthropist Daniel Ludwig, the Ludwig Institute for Cancer Research (LICR) chose the ICP in 1977 as site for its "Brussels Branch". The other LICR laboratories are located in the United States, Great Britain, Switzerland, Sweden, Australia, and Brazil. As director of its Brussels laboratory, the LICR appointed Thierry Boon, who also chairs the ICP Directorate. The laboratory has developed considerably since its creation. Today, it occupies almost one third of the ICP surface area, with a staff of about 80 investigators and technicians. Its research activities cover two main areas.

Tumor Immunology.

This group is led by Thierry Boon and comprises several subgroups including those of Aline Van Pel, Benoît Van den Eynde, Pierre van der Bruggen and Etienne De Plaen. The research is focused on the identification of antigens recognized on human tumors by cytolytic T lymphocytes of the tumor-bearing patients. Several categories of tumor antigens have been identified. An interesting group of antigens is encoded by genes that are not expressed in normal tissues but are expressed in a significant proportion of tumors of various histological types. Other antigens result from point mutations that occurred in the tumor cells.
On the basis of these findings, a program of cancer immunotherapy based on vaccination of patients with specific tumor antigens has been initiated. An European-wide collaboration between industry and several clinical centers is being organized to make it possible to test systematically many modalities of the defined antigens, such as peptide, protein or defective recombinant viruses.

Cytokines.

Directed by Jean-Christophe Renauld and Jacques Van Snick, this group is concerned with cytokines, which are proteins secreted by cells of the immune system and that control many aspects of the functions of this system. The investigators have played an important role in the discovery of interleukin 6 (IL-6), have discovered interleukin 9 (IL-9) and cloned its receptor. Current investigations deal with the *in vivo* activities of IL-9 in transgenic mice, signal transduction through the IL-9 receptor and T cell differentiation induced by this cytokine.

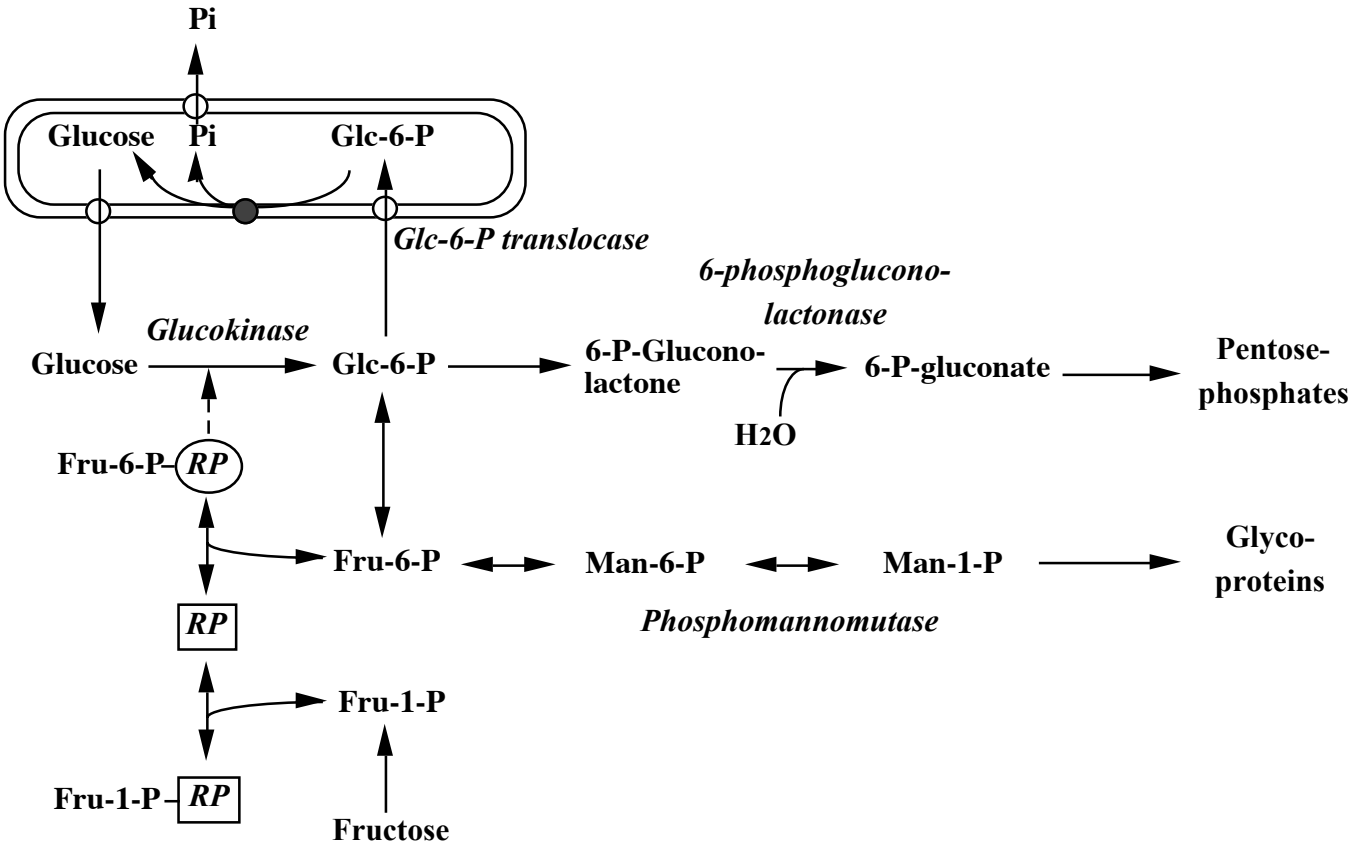
CARBOHYDRATE METABOLISM

EMILE VAN SCHAFTINGEN

Members *: Emile VAN SCHAFTINGEN, Thierry de BARSY, Henri-Géry HERS;* **Assistant Member** *: Maria VEIGA-da-CUNHA* **; Postdoctoral Fellow** *: Jean-François COLLET;* **Graduate Students** *: Younes ACHOURI, François COLLARD, Ghislain DELPIERRE, Isabelle GERIN, Moulay MOUKIL, Elsa WIAME;* **Technicians** *: Geneviève CONNEROTTE (Mrs Berghenouse), Gaëtane NOËL, Catherine PEEL;* **Secretary** *: Ilse SCHMIDT (Mrs Franklin);* **Technical Staff** *: Guy DELWICHE .*

The general goal of the group is to understand the biochemical mechanisms underlying the regulation of carbohydrate metabolism and the basis of its inherited disorders. One of our research themes is the regulation of glucokinase, the enzyme that catalyses the conversion of glucose into glucose-6-P in the liver and in the ß-cells of pancreatic islets and plays the role of a glucose sensor. In 1988 our group discovered in rat liver a regulatory protein that inhibits glucokinase competitively with respect to glucose. The effect of this protein is reinforced by fructose-6-P and antagonized by fructose-1-P (Figure) and by Pi. These effectors act by binding to the regulatory protein and by modulating its affinity for glucokinase. The effect of fructose-1-P on the regulatory protein accounts for the fact that fructose stimulates glucose phosphorylation in rat liver. Both glucokinase and its regulatory protein are nuclear proteins, at least in liver cells incubated with low concentrations of glucose. However, glucokinase translocates to the cytosol in response to fructose, sorbitol or high concentrations of glucose, whereas the regulatory protein remains in the nucleus under these conditions. The regulatory protein appears to serve as an anchor that maintains glucokinase in the nucleus when the concentration of fructose-1-P is low. Our aims are to understand at the molecular level the mechanism of regulation of glucokinase. This year we report a study on the mechanism of the positive cooperativity displayed by this enzyme. This is an important property since it sensitizes glucokinase to changes in the concentration of glucose.

The reaction opposite to that of glucokinase, the hydrolysis of glucose-6-P to glucose and Pi, is important because it provides almost all glucose needed during fasting. This reaction occurs mainly in the liver and the kidneys and is catalysed by an enzymatic system located in the endoplasmic reticulum (Figure). According to the substrate-transport model, this system comprises a hydrolase whose catalytic site faces the lumen of the organelle and various translocases responsible for the transport of glucose-6-P, Pi and glucose. Glycogen storage disease type I (GSD I) is due to glucose-6-phosphatase deficiency. The most common form, called type Ia, is caused by mutations in the gene



encoding the phosphohydrolase and a second form (GSD Ib) is due to a defect in the glucose-6-P translocase. In 1997 we reported the cloning of a human cDNA encoding a protein homologous to bacterial transporters for phosphate esters and presenting at its C-terminus a retention signal for the endoplasmic reticulum. As we found this cDNA to be mutated in GSD Ib, we concluded that it encoded the glucose-6-P translocase of the glucose-6-phosphatase system. However, there is no complete agreement on the mechanism of action of this enzyme, most particularly on the requirement of an independent glucose-6-phosphate transport and on the site of release of glucose and inorganic phosphate. The study that we report this year supports the view that the transporter for glucose 6-phosphate functions independently of the phosphohydrolase and the products of the reaction are released in the lumen of the endoplasmic reticulum.

Our long-standing interest in fructose metabolism led us recently to initiate investigations on the enzyme that forms fructose 3-phosphate in erythrocytes. As we show in our report, this enzyme can truly be considered as a fructosamine-3-kinase. This enzyme may catalyse the first step of an as yet unknown process allowing the removal of fructosamine residues.

Part of our activity is also devoted to the study of inborn errors of metabolism. In the last few years we have reported the discovery of several "new" enzymatic deficiencies.

1. MOLECULAR STUDIES ON GLUCOKINASE AND ITS REGULATORY PROTEIN

(M. Moukil, M. Veiga-da-Cunha, E. Van Schaftingen)

It has been known for more than 20 years that the saturation curve of glucokinase for glucose is sigmoidal. Since glucokinase is a monomeric enzyme that remains monomeric in the course of catalysis, classical models of cooperativity such as the symmetrical model or the sequential model are not applicable to this case. Models that better account for the kinetic behavior of glucokinase assume the existence of two different conformations with different affinities for glucose and that interconvert slowly, thus allowing glucose to increase the proportion of the conformation with higher affinity. The possibility that the cooperativity is due instead to the existence of two binding sites for glucose, with regulatory and catalytic functions, is usually ruled out on the basis of experiments indicating that, in the absence of ATP, glucose binds to glucokinase with a hyperbolic (not a sigmoidal) dependency on concentration. However, Agius and Stubbs (Biochem.

J. 346, 413-421, 2000) recently observed that mannoheptulose, a competitive inhibitor of glucokinase, is more potent to decrease the Hill coefficient of this enzyme than are other inhibitors such as N-acetylglucosamine or 5-thioglucose. This led them to postulate that mannoheptulose inhibits glucokinase by binding to a putative allosteric site for glucose. To investigate the existence of such an allosteric binding site, we have explored the stimulation exerted by glucose on fructose phosphorylation.

Using overexpressed *Escherichia coli* sorbitol-6-phosphate dehydrogenase to monitor fructose 6-phosphate formation, we found that the stimulation of fructose phosphorylation by glucose was reduced in two human ß-cell glucokinase mutants with a low Hill coefficient or when the activity of wild type glucokinase was decreased by replacing ATP by poorer nucleotide substrates. Mutation of two other residues, neighboring glucose-binding residues in the catalytic site, also reduced the affinity for glucose as a stimulator of fructose phosphorylation. Among a series of glucose analogs, only 3, all substrates of glucokinase, stimulated fructose phosphorylation; other analogs were either inactive or inhibited glucokinase. Glucose increased the apparent affinity for inhibitors that are glucose analogs, but not for the glucokinase regulatory protein or palmitoyl-CoA. These data indicate that the stimulatory effect of glucose on fructose phosphorylation reflects the positive cooperativity for glucose and is mediated by binding of glucose to the catalytic site. They support models involving the existence of two slowly-interconverting conformations of glucokinase that differ through their affinity for glucose and for glucose analogs. We show by computer simulation that such a model can account for the kinetic properties of glucokinase, including the differential ability of mannoheptulose and N-acetylglucosamine to suppress cooperativity.

2. GLUCOSE-6-PHOSPHATASE

(I. Gerin, M. Veiga-da-Cunha, E. Van Schaftingen)

According to the substrate-transport model, the glucose-6-phosphatase system comprises a relatively non-specific hydrolase, whose catalytic site is oriented towards the lumen of the endoplasmic reticulum, a specific transporter for glucose 6-phosphate and transporters for inorganic phosphate and glucose. This model accounts for several kinetic observations, including the fact that glucose-6-phosphatase acts much better on glucose 6-phosphate than on mannose 6-phosphate in intact liver microsomes, whereas it is about equally active on both substrates in detergent-treated (disrupted) microsomes. Furthermore, it allows one to rationalize the effects of chlorogenic acid and some of its derivatives (like S3483, used in this work), which inhibit the hydrolysis of glucose 6-phosphate but not of inorganic pyrophosphate in intact microsomes, while having no effect in detergent-treated microsomes. These compounds are thought to be inhibitors of the glucose 6-phosphate translocase.
The substrate-transport model also accounts for the observation that two principal types of glucose-6-phosphatase deficiency have been identified : one, known as glycogen storage disease type Ia (GSD Ia), in which the phosphohydrolase is deficient and another one (GSD Ib) in which no transport of glucose 6-phosphate can be demonstrated in liver microsomes. The genes mutated in GSD Ia and GSD Ib have been identified. The second one encodes a protein belonging to the same family as transporters (UhpT) and a putative receptor (UhpC) for hexose 6-phosphates. Although coexpression of glucose-6-phosphatase with the protein mutated in GSD Ib in COS-1 cells reconstitutes some degree of glucose 6-phosphate transport into microsomes, a clear demonstration that the protein mutated in GSD Ib acts as an independent glucose 6-phosphate translocase is still missing, owing partly to difficulties in expressing the protein in heterologous systems. Furthermore, measurements of the incorporation of radioactivity into liver microsomes incubated with radiolabeled glucose 6-phosphate show that only a fraction (at most about 10 %) of the liberated glucose or Pi can be found inside the microsomes. This led some authors to the conclusion that glucose 6-phosphate transport into the endoplasmic reticulum is not needed for its hydrolysis by glucose-6-phosphatase.

The purpose of the work we carried out last year was to discriminate between two models for glucose-6-phosphatase: one in which the enzyme has its catalytic site oriented towards the lumen of the endoplasmic reticulum, requiring transporters for glucose 6-phosphate, Pi and glucose (substrate-transport model) and a second one in which the hydrolysis of glucose 6-phosphate occurs inside the membrane (conformational model). We found that microsomes preloaded with yeast phosphoglucose isomerase catalyzed the detritiation of [2-^{3}H]glucose 6-phosphate and that this reaction was inhibited by up to 90 % by S3483, a compound known to inhibit glucose 6-phosphate hydrolysis in intact but not in detergent-treated microsomes. These results indicate that glucose 6-phosphate is transported to the lumen

of the microsomes in an S3483-sensitive manner. Detritiation by intramicrosomal phosphoglucose isomerase was up to 2-fold stimulated by 1 mM vanadate, a phosphatase inhibitor, indicating that glucose-6-phosphatase and the isomerase compete for the same intravesicular pool of glucose 6-phosphate. To investigate the site of release of inorganic phosphate (Pi) from glucose 6-phosphate, we incubated microsomes with Pb^{2+}, which forms an insoluble complex with Pi, preventing its rapid exit from the microsomes. Under these conditions, about 80 % of the Pi that was formed after 5 min was intramicrosomal, as compared to < 10 % in the absence of Pb^{2+}. We also show that, when incubated with glucose 6-phosphate and mannitol, glucose-6-phosphatase formed mannitol 1-phosphate and that this non-physiological product was initially present within the microsomes before being released to the medium. These results indicate that the primary site of product release by glucose-6-phosphatase is the lumen of the endoplasmic reticulum and support therefore the substrate-transport model.

3. FRUCTOSAMINE METABOLISM

(Gh. Delpierre, F. Collard, E. Wiame, E. Van Schaftingen, in collaboration of V. Stroobant, Brussels branch of the Ludwig Institute, Mark Rider, Hormones and Metabolism Unit, Florent Vanstapel, KULeuven and Helena Santos, Lisboa)

Fructose 3-phosphate is a phosphate ester that was initially identified in lenses from diabetic animals, where its concentration reaches ≈ 0.5 μmol/g tissue. It is also present, at lower concentrations (10-20 nmol/ml) in human erythrocytes from both control and diabetic subjects. Formation of fructose 3-phosphate can be induced by incubating erythrocytes or lenses with fructose, the apparent K_M for this substrate being about 30 mM in erythrocytes. Furthermore, erythrocyte lysates have been reported to catalyse the phosphorylation of fructose on its third carbon in the presence of ATP, but the kinase responsible for this reaction has not been further characterised or purified, except for a brief mention in an abstract that fructoselysine could also be a substrate. The function of fructose 3-phosphate is unclear, as no enzyme that can consume this compound has been identified. The low affinity for fructose of the enzyme that forms fructose 3-phosphate and the absence of a satisfying role for this phosphate ester suggested that the physiological substrate of fructose-3-kinase may be not fructose itself but structurally related compounds, possibly fructosamines.

Fructosamines are the products of a non-enzymatic reaction of glucose with primary amines followed by an Amadori rearrangement. These reactions, known as glycation (to be distinguished from glycosylation, which is an enzymatically-catalysed process), can typically modify the amino terminus and the lysine and arginine side-chains of proteins, as well as a variety of low-molecular-weight compounds, including aminophospholipids.

To identify the potential physiological substrate of fructose-3-kinase, we tested the effect of several sugars or sugar derivatives on the formation of fructose 3-phosphate in human erythrocytes. We found that this formation (measured in the presence of 50 mM fructose) was inhibited by deoxymorpholinofructose (DMF), a synthetic fructosamine, with an apparent Ki of 100 μM. ^{31}P-NMR analysis of extracts of cells incubated with DMF indicated the presence of an additional phosphorylated compound, which was partially purified and shown to be DMF 3-phosphate by tandem mass spectrometry. Radiolabelled DMF was phosphorylated by intact erythrocytes with an apparent K_M (≈ 100 μM) about 300-fold lower than the value reported for fructose phosphorylation on its third carbon. These results indicated that the physiological function of the enzyme able to convert fructose to fructose 3-phosphate in intact erythrocytes is most likely to phosphorylate fructosamines.

We next undertook the purification of fructosamine 3-kinase. Erythrocyte extracts were found to catalyze the ATP-dependent phosphorylation of DMF. The enzyme responsible for this conversion was purified about 2500-fold by chromatography on Blue Sepharose, Q Sepharose and Sephacryl S-200, and shown to copurify with a 35,000-M_r protein. Partial sequences of tryptic peptides were derived from the protein by nanoelectrospray-ionization mass spectrometry, These partial sequences allowed the identification of the corresponding human and mouse cDNA's. Both cDNA's encode proteins of 309 amino acids, showing 89% identity with each other and which are homologous to proteins of unknown function predicted from the sequences of several bacterial genomes. Both proteins were expressed in *E. coli* and purified. They were shown to catalyze the phosphorylation of DMF, fructoselysine, fructoseglycine and fructose in order of decreasing affinity. They also phosphorylated glycated lysozyme, though not unmodified lysozyme. NMR analysis of phosphorylated DMF and phosphorylated fructoseglycine showed that the phosphate was bound to the third carbon of the 1-deoxyfructose

moiety. The physiological function of fructosamine-3-kinase may be to initiate a process leading to the deglycation of fructoselysine and of glycated proteins.

4. CARBOHYDRATE-DEFICIENT GLYCOPROTEIN SYNDROME

(J.F. Collet, E. Van Schaftingen, in collaboration with S. Grünewald, J. Jaeken, G. Matthijs, E. Schollen, KULeuven)
Congenital disorders of glycosylation (CDG) are a rapidly enlarging group of inherited diseases with abnormal N-glycosylation of glycoconjugates. Most patients belong to CDG-Ia due to a phosphomannomutase (PMM) deficiency (see ICP report 1996). We have now found that a significant part of CDG-Ia patients (9 out of 54) had a rather high residual PMM activity in fibroblasts amounting to 35–70% of the mean control value and included in the normal range (defined as means of the controls ± 2 SD). The clinical diagnosis of CDG-Ia was made difficult by the fact that most of these patients (6/9) belong to a subgroup that is characterized by a milder phenotype than classical CDG-Ia patients. These patients lack some of the symptoms that are suggestive for the diagnosis such as inverted nipples and abnormal fat deposition and, as a mean, had higher residual PMM activities in fibroblasts (2.05 ± 0.61 mU/mg protein, n=9; controls 5.34 ± 1.74, n=22), compared to moderate (1.32 ± 0.86 mU/mg protein, n =18) or severe (0.63 ± 0.56 mU/mg protein, n=27, $P < 0.001$) cases. Yet they all showed mild mental retardation, hypotonia, cerebellar hypoplasia and strabismus. All of them had an abnormal serum transferrin pattern and a significantly reduced PMM activity in leukocytes. Six out of the nine mild cases were compound heterozygotes for the C241S mutation, which is known to reduce PMM activity by only about 2-fold. Our results indicate that intermediate PMM values in fibroblasts may mask the diagnosis of CDG-Ia, which is better done by measuring PMM activity in leukocytes and mutation search in the *PMM2* gene. They indicate also that there is some degree of correlation between the residual activity in fibroblasts and the clinical phenotype.

5. REGULATION OF SERINE BIOSYNTHESIS

(Y. Achouri, M. Robbi, E. Van Schaftingen, in collaboration with C. Szpirer, ULB)

We have determined the structure of the rat gene encoding 3-phosphoglycerate dehydrogenase, an enzyme of the serine biosynthesis pathway, whose expression in the liver, but not in other tissues, is negatively controlled by cysteine. The gene contains 13 exons, spans ≈ 30 kb and is located on rat chromosome 2q34, a region syntenic to human chromosome 1p. 5'-RACE experiments indicated that the same promoter is used in liver as in other tissues but that a second promoter, about 200 bp more upstream, is also used in testis. Transfection of hepatoma cells with various portions of the putative ubiquitous promoter showed that it drove the expression of a reporter gene in a cysteine-insensitive manner. This is in agreement with previous results indicating that the sensitivity of 3-PGDH expression to this amino acid is mediated by changes in mRNA stability.

6. INBORN ERRORS OF METABOLISM

(T. de Barsy, E. Van Schaftingen)

In 2000, samples from about 120 patients were analysed, allowing the diagnosis of carnitine deficiency (1 case), of carnitine palmitoyl transferase deficiency (1 case), of various forms of glycogen storage disease (7 cases), of phosphomannomutase deficiency (18 cases) and phosphomannose isomerase deficiency (1 case).

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PURINE METABOLISM

GEORGES VAN DEN BERGHE

Member : *Georges VAN DEN BERGHE*; **Associate Members** *: Françoise BONTEMPS (Mrs Michotte), Marie-Françoise VINCENT (Mrs Lambin);* **Postdoctoral Fellows** *: Sandrine MARIE (Mrs André), Eric VAN DEN NESTE;* **Graduate Students** *: Sabine CARDOEN, Valérie RACE, Caroline SMAL;* **Technicians** *: Anne DELACAUW (Mrs Jones), Thérèse TIMMERMAN;* **Technical Staff** *: Guy DELWICHE(till September), Cédric VERELST (from September);* **Secretary** *: Ilse SCHMIDT (Mrs Franklin).*

The major interests of this group are the regulation of purine metabolism, its genetic defects, and the pharmacologic and therapeutic effects of select purine nucleoside analogues.

Purine metabolism comprises three major pathways (Figure). The ten-step synthetic route (not shown in detail), often termed 'de novo', leads from phosphoribosyl pyrophosphate (PRPP) to IMP. From IMP, the nucleoside monophosphates, AMP and GMP, and the corresponding di- and triphosphates (not shown) are formed. The catabolic pathway starts from the nucleoside monophosphates and, in man, produces uric acid, a poorly souble compound. In lower mammals, uricase (not shown) converts uric acid into allantoin, which is much more soluble. The salvage pathway, composed of two enzymes, hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase, converts the purine bases, guanine, hypoxanthine and adenine, into the corresponding nucleoside monophosphates. Adenosine kinase can also be considered a salvage enzyme.

Purine catabolism should be kept to a minimum for two reasons: (1) to preserve the nucleotide pools which play an essential role in cell metabolism; (2) in addition in man, to limit the production of uric acid so as to avoid its precipitation with the damaging consequences found in gout.

Our initial work has led to the conclusion that, in several tissues, the catabolism of AMP, leading to the production of uric acid and/or allantoin, proceeds only by way of AMP deaminase, a highly regulated enzyme which is normally more than 95 % inhibited. Dephosphorylation of AMP into adenosine also occurs but does not contribute to the formation of purine catabolites, owing to recycling by way of adenosine kinase. We have also shown that interruption of this recycling plays a major role in the elevation of adenosine, a major regulatory compound, under anoxic conditions. We furthermore demonstrated that a variety of rat and human tissues contain a cytosolic 5'-nucleotidase which hydrolyzes preferentially IMP and GMP, is stimulated by ATP and 2,3-bisphosphoglycerate, and inhibited by Pi.

Collaboration with the Department of Paediatrics of the University Hospital Gasthuisberg in Leuven has led us to the discovery, in 1984, of adenylosuccinase (adenylosuccinate lyase, ADSL) deficiency, the first enzyme deficiency described on the 'de novo' pathway of purine synthesis in man. This disorder causes accumulation in cerebro-spinal fluid and urine of two normally undetectable compounds, succinyladenosine (S-Ado) and succinylamino-imidazolecarboxamide riboside (SAICAriboside). These are the dephosphorylated derivatives of the two substrates of ADSL, adenylosuccinate (S-AMP) and SAICAribotide (SAICAR), respectively. Affected children display variable, but mostly profound

psychomotor delay, often epilepsy and/or autistic features, occasionally growth retardation and muscular wasting. More recently, studies of the molecular biology of ADSL deficiency have been initiated.

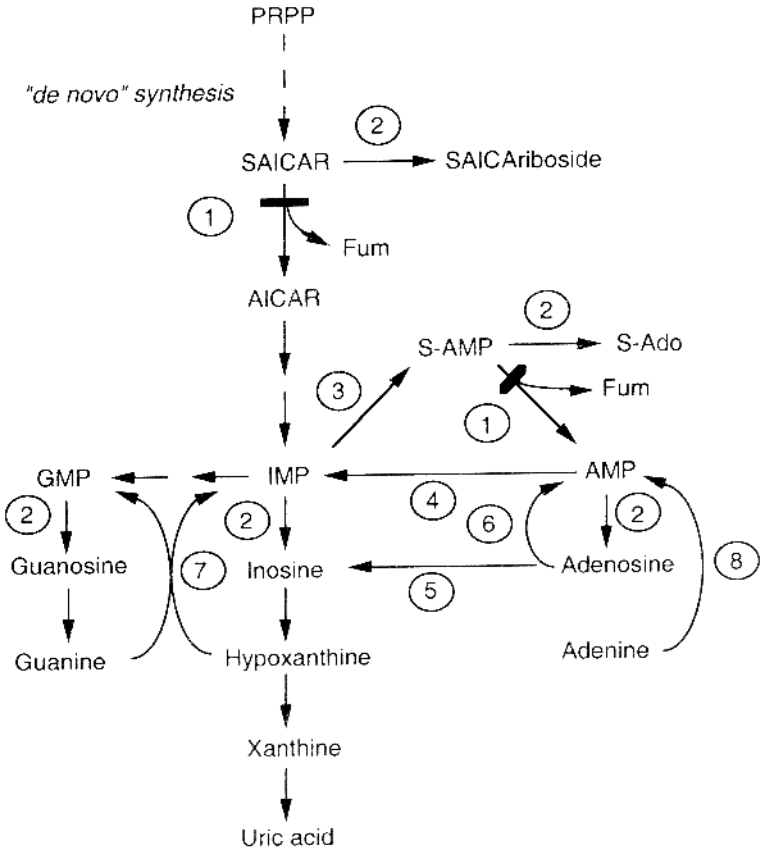

Figure. Pathways of purine metabolism.
AICAR, aminoimidazolecarboxamide ribotide; Fum, fumarate, PRPP, phosphoribosyl pyrophosphate; S-Ado, succinyladenosine; SAICAR, succinylaminoimidazolecarboxamide ribotide; S-AMP, adenylosuccinate. (1) adenylosuccinase; (2) cytosolic 5'-nucleotidase; (3) adenylosuccinate synthetase; (4) AMP deaminase; (5) adenosine deaminase; (6) adenosine kinase; (7) hypoxanthine-guanine phosphoribosyltransferase; (8) adenine phosphoribosyltransferase.The adenylosuccinase defect is depicted by solid bars.

Studies of the pathogenesis of ADSL deficiency have revealed that a related compound, AICAriboside, inhibits glycolysis, gluconeogenesis, and the synthesis of both fatty acids and cholesterol in the liver. These effects were shown to be due to phosphorylation of AICAriboside into AICAR. This compound, also termed ZMP, inhibits key glycolytic and gluconeogenic enzymes owing to its resemblance with AMP. Moreover, it stimulates AMP-activated protein kinase. This provokes phosphorylation and inactivation of acetyl-CoA carboxylase and HMG-CoA reductase, the rate-limiting enzymes of fatty acid and cholesterol synthesis, respectively.

In 1997, a collaborative study of the antileukaemic nucleoside, 2-chloro-2'-deoxyadenosine (CdA), was started with the Department of Haematology of the University Hospital Saint-Luc. This adenosine deaminase-resistant adenosine analogue displays remarkable therapeutic properties in chronic lymphoid malignancies. Nevertheless, resistance is also observed, and CdA does not confer a survival advantage when compared to more conventional

therapies such as alkylating agents. The aims of the project are to understand the mechanisms that lead to resistance to CdA, and to improve its therapeutic efficacy by searching for synergisms with other anticancer compounds.
In 2000, we have pursued studies of the mutations and pathogenesis of adenylosuccinate lyase deficiency, and of the antileukaemic properties and mechanism of action of 2-chloro-2'-deoxyadenosine. An investigation of the regulation of deoxycytidine kinase (dCK), a key enzyme in the activation of several therapeutic nucleoside analogues, has also been initiated.

1. ADENYLOSUCCINATE LYASE DEFICIENCY

(S. Marie, V. Race, T. Timmerman, M.-F. Vincent)

Mutation analysis

ADSL deficiency has been diagnosed in more than 50 patients worldwide. In accordance with the variability of the clinical picture, 27 different mutations in the ADSL gene have been identified to date in 28 unrelated families. The majority are missense mutations, with the exception of a splicing error leading to deletion of 39 bases at the end of exon five in the mRNA sequence (Marie et al, Hum Mutat 13: 197-200, 1999; Van den Berghe & Jaeken, in The Metabolic and Molecular Bases of Inherited Disease [CR Scriver, AL Beaudet, D Valle, WS Sly, eds], 8th ed, pp 2653-2662, McGraw Hill, New York, 2001). In about half of the families, the patients are compound heterozygotes. Most frequently encountered, accounting for about one third of the alleles investigated, is a R426H mutation, which has been found in the homozygous form in 9 families, and in the compound heterozygote form in 4 families.

Last year, we also found a c.49T→C change in the 5' untranslated region (5'UTR) of the ADSL gene, in association with the R426H mutation, in an Italian-American girl with ADSL deficiency. Studies of the influence of this mutation on gene expression showed that it provokes a 80 % reduction of the capacity of the promoter region, containing a 400 bp sequence upstream to the initiation ATG, to lead to expression of luciferase activity. Measurements of luciferase mRNA levels showed that the amount of messenger was also reduced 3- to 4-fold with the mutated construction.

Further analysis has now shown that the mutation is located within a potential binding site for transcriptional factor NRF-2. By site-directed mutagenesis we could demonstrate that another mutation within the core sequence of this site has the same effect as c.49T→C. However, gel-shift assays showed that 3 different DNA-protein complexes are formed with wild-type ADSL, mutated ADSL, and NRF-2 site oligonucleotides, respectively.

The results suggest that the mutation in the 5'UTR either prevents the binding of an unknown activating factor, or allows the binding of a repressing factor, causing a reduction of the level of transcription.

Pathogenetic studies

The symptoms of ADSL deficiency could a priori be due either to a deficiency of purine, particularly adenine nucleotides, or to a toxic effect of the accumulating succinylpurines, S-Ado and SAICAriboside. Measurements performed hitherto have shown normal nucleotide levels in ADSL-deficient tissus that may be explained by the presence of residual enzyme activity and/or operation of the salvage pathway. The observation that the levels of SAICAriboside are comparable in severely and mildly retarded patients, whereas those of S-Ado are markeldly higher in the latter, has led to the hypothesis that SAICAriboside is the neurotoxic compound, and that S-Ado could counteract its noxious effects.

The structural similarity of the succinylpurines with *N*-acetylaspartate (NAA), and with *N*-methyl-D-aspartate (NMDA), has prompted studies of their potential interference with the metabolism of NAA and with the receptors for NMDA. NAA accumulates in Canavan (or van Bogaert-Bertrand) disease owing to the deficiency of aspartoacylase, and is thought to be the offending compound in this devastating neurodegenerative disorder. Inhibition of aspartoacylase might thus be particularly noxious. However, measurements of the activity of the enzyme in rat brain and liver, and in human fibroblasts, showed that it was not influenced by up to 1 mM concentrations of SAICAriboside and S-Ado.

In neurons, excessive stimulation of NMDA receptors can lead to excitotoxicity, caused by inordinate entry of calcium inside the cells. To assess whether SAICAriboside and/or S-Ado could stimulate NMDA receptors, their effects were investigated in primary cultures of brain cortical neurons, prepared from 17 day-old rat embryos. After 10 days of culture to allow cellular differentiation, entry of calcium was measured after a 1 hour incubation with 2 μM Fura 2AM, followed by

fluorescence recording with a digital multiparametric video microscope. Whereas the principal excitotoxic compound, glutamate, and NMDA provoked a marked elevation of intraneuronal calcium, neither SAICAriboside nor S-Ado, at up to 5 mM concentration, had any effect. Like that of many inborn errors of metabolism, the pathogenesis of ADSL deficiency remains elusive.

2. ANTILEUKAEMIC PROPERTIES OF 2-CHLORO-2'-DEOXYADENOSINE

(F. Bontemps, S. Cardoen, A. Delacauw, C. Smal, E. Van Den Neste, in collaboration with A. Ferrant, Department of Haematology, University Hospital Saint-Luc)

2-Chloro-2'-deoxyadenosine (CdA), an adenosine deaminase resistant analogue of deoxyadenosine, has major therapeutic properties in indolent lymphoid malignancies, including hairy cell leukemia and B-cell chronic lymphocytic leukemia (B-CLL). To exert is antileukemic effect, CdA has to be phosphorylated by deoxycytidine kinase (dCK) into CdAMP, followed by conversion into CdADP and CdATP. The latter, the active metabolite of CdA, has been shown to interfere with a variety of enzymes involved in DNA synthesis and repair, including ribonucleotide reductase and DNA polymerases α and β.Moreover, CdATP can be incorporated into newly synthesized DNA, causing chain termination. Together, these actions result in the progressive accumulation of DNA strand breaks, leading to initiation of apoptosis by mechanisms which are not yet entirely clear.

To improve our understanding of the mechanisms by which CdA induces apoptosis in B-CLL cells, we investigate EHEB cells, a continous cell line derived from a patient with B-CLL. With the aim to optimize the conversion of CdA and other nucleoside analogues into their active, triphosphate form, we study the regulation of dCK, an enzyme with broad specificity which phosphorylates numerous nucleoside analogues used in anticancer and antiviral therapy.

Studies of resistance to CdA in EHEB cells

This cell line was found to be less sensitive to CdA than B-CLL lymphocytes (~ 25-fold) and other human lymphoblastic cell lines (10- to 1000-fold). Phosphorylation of CdA by dCK, as well as intracellular accumulation of CdATP, which has often been found reduced in CdA-resistant cells, were similar in EHEB cells and in other CdA-sensitive cell lines. In contrast, the inhibitory effect of CdA on ribonucleotide reductase activity, investigated in intact cells by incorporation of labeled cytidine (Cyd) into DNA, was much less pronounced in EHEB cells than in other human lymphoblastic cells. Accordingly, incorporation of thymidine (dThd) into DNA was only weakly inhibited by a 4-h incubation in the presence of up to 10 μM CdA. Moreover, after a 24 h-incubation, it was unexpectedly stimulated, up to 2-fold. An even greater stimulatory effect of CdA was recorded on deoxycytidine (dCyd) incorporation into DNA. Analysis of cell cycle by flow cytometry, using double labelling with propidium iodide and bromodeoxyuridine, showed that CdA induces both a significant increase in the proportion of cells in S phase and of cells synthesizing DNA. CdA also increased approximately 4-fold the activities of both dCK and thymidine kinase (TK), and tended to elevate the concentrations of the deoxynucleoside triphosphates (dNTPs). We conclude that the EHEB cell line is resistant to the cytotoxic action of CdA, not only because of a near absence of inhibition of ribonucleotide reductase, but also because CdA provokes in this cell line an increase in the proportion of cells replicating their DNA, an effect which is at variance with the known effect of CdA.

Regulation of dCK activity

Activation of dCK by CdA was found not to be due to an increased synthesis of the enzyme. To unravel the mechanism of this activation, we investigated the effect of a large variety of activators and inhibitors of protein kinases in EHEB cells. Genistein, a phytoestrogen from soybeans with a flavonoid chemical structure, widely used to inhibit protein tyrosine kinases and topoisomerase II, was found to increase 2- to 3-fold the activity of dCK. The increase was time- and dose-dependent, with a maximum after 4 h of incubation and 30 μg/ml of genistein. Western blots showed that the increased dCK activity was not due to an enhancement of the synthesis of the enzyme protein. Persistence of the increased activity after filtration on Sephadex G25 indicated that it was caused by a stable modification of the enzyme. Other inhibitors of topoisomerase II were also found to activate dCK, although to a lesser extent than genistein. AG-490, a synthetic tyrphostin with an inhibitory effect on the protein tyrosine kinase JAK, was also active. In contrast, neither herbimycin A, another inhibitor of cytoplasmic protein tyrosine kinases, nor wortmannin, a specific inhibitor of

PI3kinase, influenced the activity of dCK. Further studies are in progress to verify if CdA and genistein act by a common intracellular signalling pathway.

Effect of UV light in B-CLL cells

Studies in B-CLL cells showed that UV-C radiation (ranging from 3.3 to 30 J/m²) dose-dependently increased the incorporation into DNA of [³H]dCyd, to a maximum of 776 ± 151% of control at 20 J/m², whereas the increase in [³H]dThd uptake was less marked (median: 207 ± 82% at 20 J/m²). Assessment of the influence of UV light on the activities of dCK and TK in cell extracts over 3 h following UV radiation, showed a time- and dose-dependent upregulation of dCK activity. The maximal stimulation was observed 30 min after 30 J/m² of UV (256 ± 44% of control). In contrast, TK activity was not augmented after UV radiation. The upregulation of dCK activity remained unchanged after dialysis of the extracts. A similar extent of dCK activity upregulation was observed in identical conditions in EHEB cells and in CEM cells, a continous lymphoblastoid cell line.
Like its activation by CdA, the activation of dCK by UV light, which appears to occur in resting as well as in dividing cells, might be due to post-translational modifications of the enzyme. Our results justify therapeutic trials combining nucleoside analogues that are phosphorylated by dCK, and UV irradiation that can be delivered to patients by extracorporeal photophoresis.

3. OTHER INBORN ERRORS OF METABOLISM

(T. Timmerman, M.-F. Vincent)

In 2000, 598 samples were analysed for the diagnosis of inborn errors of metabolism by measurements of accumulating metabolites (purines and pyrimidines, mucopolysaccharides, sialic acid, intra-leukocytic cystine) and/or various enzyme activities. Cystinosis was diagnosed in two patients. Each of the following diseases was diagnosed in one patient: mucopolysaccharidosis type I, Tay-Sachs disease, metachromatic leukodystrophy, classical galactosaemia, galactokinase deficiency, Gaucher disease, Fabry disease, Niemann-Pick disease, mucolipidosis type III, cystinosis, and ADSL deficiency. Mutation analysis was performed in 5 ADSL-deficient patients from France, Germany, Norway, Spain, and The Netherlands. Five new mutations were found. We also set up the measurement of mevalonate kinase activity in lymphoblasts, and participated in a study of this enzyme in patients suffering of the hyper-IgD and periodic fever syndrome.

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HUMAN MOLECULAR GENETICS

MIIKKA VIKKULA

Associate Member *: Miikka VIKKULA; Part time* **Research Associate** *: Laurence BOON;* **Postdoctoral Fellow :** *Iiro EEROLA;* **Graduate Students** *: Pascal BROUILLARD, Ilse GUTIERREZ-ROELENS, Alexandre IRRTHUM, Emmanuel ROUSSEAU; Brendan MCINTYRE;* **Technician** *: Ana GUTIERREZ;* **Secretary***: Liliana NICULESCU.*

The basic aim of our research is to get insights into the molecular mechanisms behind various inherited diseases. We are especially interested in disorders affecting the cardiovascular and the skeletal system. In addition, we have initiated studies on some cancerous tumors. As this research is based on human DNA extracted from blood and tissue samples obtained from patients, the group works tightly together with several clinicians and multidiciplinary centers worldwide (e.g. Centre des Malformations Vasculaires, Cliniques Universitaires St-Luc; Vascular Anomalies Center, Children's Hospital, Boston, USA and Centre labiopalatin, Cliniques Universitaires St-Luc).

1. VENOUS MALFORMATIONS AND VENOUS MALFORMATIONS WITH "GLOMUS" CELLS ("GLOMANGIOMAS")

(P. Brouillard, A. Irrthum, B. McIntyre, L. Boon and M. Vikkula, in collaboration with B.R. Olsen, Harvard Medical School, Boston, USA; J.B. Mulliken and S. Fishman, Children's Hospital, Boston, USA and O. Enjolras, Hôpital Lariboisière, Paris, France)

Venous malformations (VM) are bluish-purple cutaneous and mucosal lesions. They are often congenital, but can also appear later in life. They have a tendency to grow slowly with the growth of the child. Glomuvenous malformations are a special subtype of venous anomalies. They are clinically similar to VMs. In histologic examination, the pathognomonic sign is the appearance of "glomus cells" around the endothelium lining the convoluted channels. These cells are thought to be smooth muscle cells of origin.

We have previously identified that hereditary venous malformations can be caused by an activating mutation in the endothelial specific receptor tyrosine kinase *TIE2/TEK* (Vikkula et al. Cell 1996; 87:1181-90). Also, we showed with immunohistochemical analysis that VMs have a reduced number of smooth muscle cells surrounding the endothelium. Mice homozygous for Tie2/Tek null allele die at E9.5 due to dysmorphogenesis of the developing vasculature. As the gain-of-function effect of the human mutation is opposite to this loss-of-function effect introduced into the mouse, we are creating a transgenic mouse mimicking the human condition in collaboration with Prof. Olsen (Harvard Medical School, Boston).

In contrast to VMs, glomuvenous malformations have a high number of smooth muscle cell-like "glomus" cells around the affected venous-like lumens. This feature suggests that the etiopathogenic mechanisms of VMs and "glomangiomas" are related, yet different. By studying several families with inherited glomuvenous malformations, we identified a 4-6 cM chromosomal region, *VMGLOM,* linked to the phenotype (Boon et al., Am J Hum Gen 1999; 65:125-133). We excluded three positional candidate genes by SSCP and heteroduplex analyses, and Southern blot hybridizations. To identify the *VMGLOM* gene, we obtained a YAC (yeast artificial chromosome) contig covering the whole 5 Mbp VMGLOM locus (Figure 1 and cover). In addition, we collected samples from several other families, and identified linkage disequilibrium between certain markers and the phenotype (Figure 2), (Irrthum et al., Eur J Hum Genet, in press). This allowed us to narrow the locus to 1.48 Mbp, covered by a single YAC (Figure 1)

(Brouillard et al., Genomics, 2000; Irrthum et al., Eur J Hum Genet, in press). Using this single YAC as template, we created a PAC (P1 bacterial artificial chromosome) contig for this reduced VMGLOM region (Brouillard et al., Genomics, 2000). This high-resolution physical map allowed us to precisely localize expressed sequences, and thus genes, into the locus. Characterization of these novel candidate genes has recently lead to the identification of the mutated gene, that we named "glomulin" (Brouillard et al., submitted for publication).

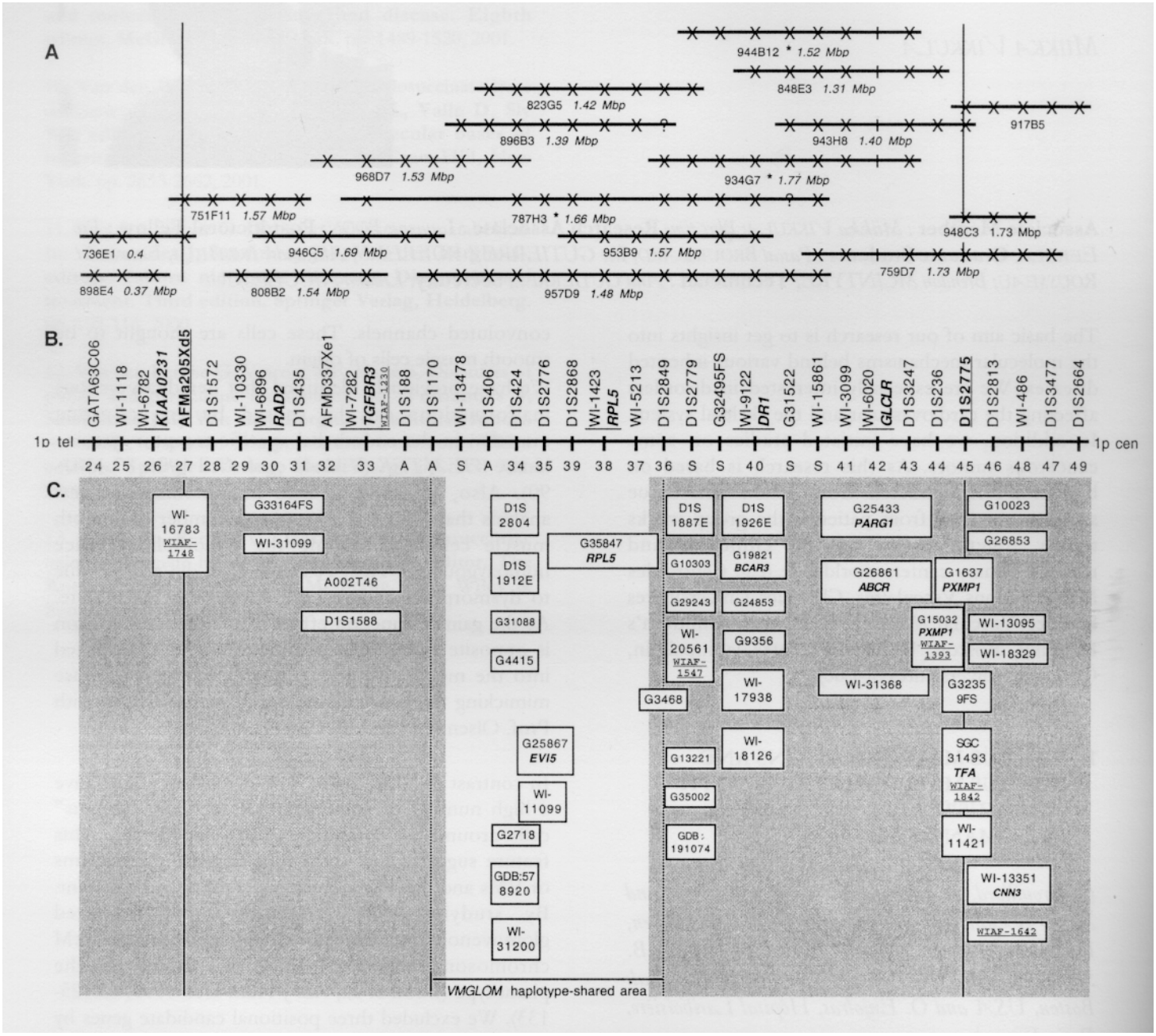

Figure 1. Schematic representation of the YAC map of VMGLOM and STS localization. *Genes marked in bold, italic, SNPs in small underlined capital letters (WIAF).* **A***) * = YAC clone reported to be chimeric. Results for marker WI-6020 marked with* **I** *to reflect database entries only. ? = unclear results for marker D1S2849 for YAC 896b3.* **B***) Numbers under markers correspond to WC1.14 contig from Whitehead/MIT database. A = from (Allikmets et al. 1997). S = placed during STSs localization. Order for markers #36 to #39 is inverted to reflect order in PAC map.* **C***) Boxes represent areas of localization for mapped or novel STSs. Vertical lines delimit VMGLOM locus (unbroken lines) or smaller, haplotype-shared area (dashed lines).*

	HAPLOTYPE A										
	Bl USA	Bt Bel	Sh USA	F Sco	T Ita	Bln Fra	Sch Ger	n/N fam.	n/N con.	P	
A205XD5	241	239/R	241/245	245/R	241	241	245	-	-	-	
D1S435	175	157	161	175	175	157	157	5/12	25/60	1.000	
B337XE1	263	263	267	281	259	263	267	4/12	24/54	0.481	
D1S188	162	168	166	166	166	168	166	3/12	6/54	0.205	
D1S1170+	118	118	118	118	122	118	118	10/12	30/60	0.034	
33CA1	156	156	156	156	152	156	156	9/12	18/62	0.002	*
D1S2804	179	179	179	179	179	179	179	7/12	10/62	0.001	*
D1S424	223	225	223	223	223	225	223	5/12	9/62	0.033	
D1S406+	200	200	200	204	200	196	200	5/12	6/64	0.003	*
69CA1	171	169/171	171	171	171	171	171	9/12	15/60	0.001	*
D1S2776	206	206	206	206	206	206	206	8/12	8/62	0.000	*
50CA1	127	125/127	129	127	127	127	127	7/12	2/48	0.000	*
D1S2868	146	146	146	146	146	146	146	7/12	14/62	0.012	
75CA1	171/173	171/173	173	173	171	173	173	6/12	5/54	0.001	*
D1S2849	179	179	179	179	179	179	179	8/12	17/64	0.007	*
D1S2779	229	231	233	231	241	231	231	4/12	21/62	0.971	
D1S236	194	210	194	190	212	190/R	190/R	3/12	37/62	0.027	
D1S2775	201/R	199/201	201	201	201	195/R	201/R	-	-	-	

Figure 2. Haplotype A sharing in VMGLOM. *Numbers indicate sizes of alleles that segregate with the disease in each family. At the top, symbol and geographic origin of family. USA: The United States of America; Bel: Belgium; Sco: Scotland; Ita: Italy; Fra: France; Ger: Germany. +: tetranucleotide repeat microsatellite. R: a recombinant individual in the family for this marker. X/Y: data not informative for linked allele. Alleles with a probable ancestral mutation differ from shared haplotype: white background. n/N: number of the shared allele on total number of alleles. fam: families linked to VMGLOM. con: control individuals from the Belgian population. P: P-value for the uncorrected chi-square test in a 2x2 table. *: significant P-value (p<0.01).*

2. VASCULAR ANOMALIES AFFECTING CAPILLARIES

(I. Eerola, LM. Boon and M. Vikkula in collaboration with J.B. Mulliken, Children's Hospital, Boston, USA, S. Watanabe, Showa University School of Medicine, Tokyo, Japan, A. Dompmartin, CHU de Caen, France and K.H. Plate, Freiburg, Germany)

Capillaries, the small blood vessels that connect arterioles to venules, can give rise to various anomalies, two of which are very common: 1) hemangioma, a benign, localized overgrowth of capillary-like vessels, and 2) capillary malformation (CM; commonly known as portwine stain), a lozalised maldevelopment of capillary like vessels. Hemangiomas have a frequency up to 12% in 1-year-old children, and CMs occur in 0,3% of newborns. Whereas hemangiomas usually disappear spontaneously, capillary malformations stay throughout life, if not treated. Other types of cutaneous capillary anomalies also exist, in addition, some can affect other organs, like CCMs, cerebral capillary malformations.

As the molecular mechanisms leading to these localized capillary lesions are unknown, we have started to collect clinical information and samples from families in which more than two individuals are affected. With these studies, we discovered that inherited hyperkeratotic cutaneous capillary-venous malformations (HCCVM) associated with cerebral capillary malformations are caused by a mutation in the *KRIT1* (Krev interaction trapped 1) gene (Eerola et al, Hum Mol Gen, 2000). This suggests that KRIT1, a possible intracellular signaling molecule, is important not only for cerebral but also for cutaneous vasculature. As our Northern-hybridisation results showed that the KRIT1 transcript is bigger than expected, we started a project to verify the length of the KRIT1 cDNA. Using *in silico* cloning we identified 8 additional exons, four of which are also translated (Eerola et al., in press) (Figure 3). These newly identified exons are important for allowing analysis of a more complete KRIT1 gene in patients with CCMs and/or HCCVMs. Their identification also enables KRIT 1 expression studies.

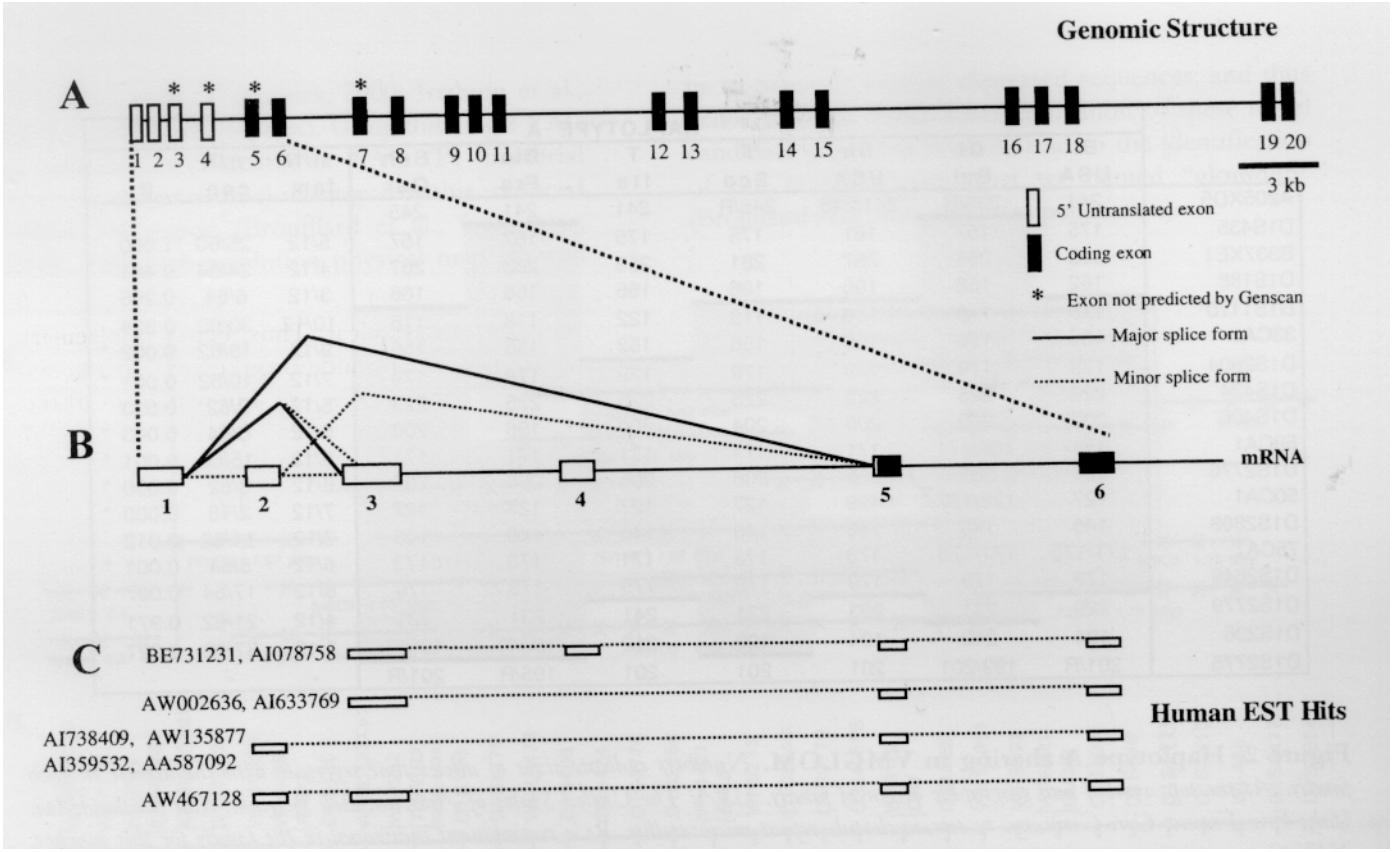

Figure 3. KRIT1 gene. *A. Overall genomic structure of the CCM1 gene encoding KRIT1. B. Alternative splicing of the 5'-end of the gene based on RT-PCR results; C. The longest human ESTs similar to the 5'-end of the gene.*

2. OTHER VASCULAR ANOMALIES

3. Irrthum, L. Boon and M. Vikkula, in collaboration with K. Devriendt, KUL and J.B. Mulliken, Children's Hospital, Boston, USA)

In addition to the malformations already described, other, heritable and non-heritable, vascular and/or combined malformations exist. Congenital lymphedema, a chronic swelling of lower extremities that causes dysfunction and frequent infections, is sometimes inherited.

We have studied such a family and identified that the phenotype is linked to chromosome 5q. In addition, mutational screenings led to the identification of a single nucleotide change that causes an amino acid substitution in the vascular endothelial growth factor receptor 3 gene (VEGFR3) (Irrthum et al., Am J Hum Gen, 2000) (Figure 4a). The localization of the amino acid substitution at a position conserved between tyrosine kinase receptors suggests that the mutation leads to loss of function. In fact, expression studies which compared the autophosphorylation status of the wild-type and the mutant receptor, showed that this is the case (Figure 4b). Thus, intervention with VEGFR3 signaling may be a new way to treat lymphedema.

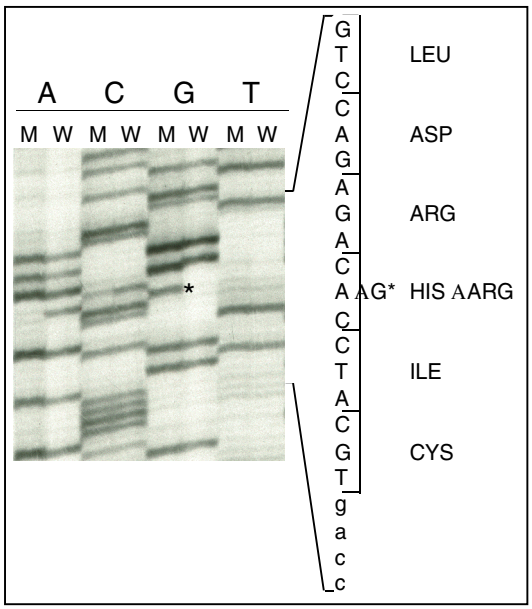

Figure 4a - Autoradiogram showing sequences of two clones corresponding to wild-type (W) and mutant (M) alleles in individual I.1. *Small letters, intronic sequence; capital letters, exonic sequence;*,mutation.*

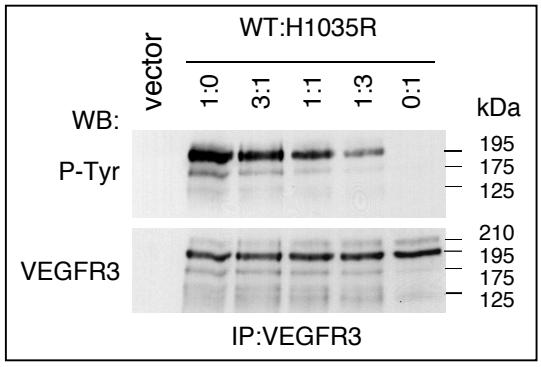

Figure 4b - Effect of H1035R mutation on tyrosyl autophosphorylation of VEGFR3. *Expression plasmids for WT and mutant VEGFR3 were transfected into 293T human embryonic kidney cells (lanes 1:0 and 0:1, respectively), or cotransfected using 3:1, 1:1 and 1:3 ratios of WT to mutant plasmid. Receptor phosphorylation was analyzed from VEGFR3 immunoprecipitates by Western blotting using anti-phosphotyrosine antibodies (P-Tyr, upper part). Total amount of receptor protein is shown in VEGFR3 Western blotting (lower part). Sizes on the right correspond to four different forms of* VEGFR3.

4. CARDIOPATHIES

(I. Gutierrez-Roelens, A. Irrthum and M. Vikkula, in collaboration with T. Sluysmans, St-Luc, UCL and K. Devriendt, KUL)

The cardiovascular system may encounter developmental problems affecting the heart too. These cardiac defects, cardiopathies, vary from physiological septal defects to life-threatening complex malformations. To get insight into the molecular mechanisms behind these phenotypes, we have started to collect samples from families with a possible hereditary cardiopathy.

5. OSTEOARTHROSIS AND OSTEOCHONDRODYSPLASIAS

(M. Vikkula, in collaboration with D. Manicourt, and B. Bayet and R. Vanwijck, St-Luc, UCL)

Our main project is in collaboration with Centre labio-palatin, St Luc, to unravel the molecular background of non-syndromic cleft lip and/or palate. Numerous blood samples of affected individuals, and their parents and siblings were collected during the year. This will be continued so as to allow us to do association studies in the future.

6. CEREBRAL TUMORS

(E. Rousseau and M. Vikkula, in collaboration with C. Godfraind, Laboratory of Neuropathology, St-Luc, UCL)

Morphological characterization and classification of tumors is not always clear. Thus, better (molecular) criteria are needed. In addition, the causative genes are often unknown. We are interested in three cerebral tumors : neurocytoma, ependymoma, and oligodendroglioma/astrocytoma.We have set up the conditions for using DNA, extracted from formalin-fixed and paraffin-embedded tissues, for genome-wide loss-of heterozygosity screening. A complete screen for neurocytomas was performed without identifying lost regions (Godfraind et al., in preparation). In addition, a similar screening was performed in a number of oligodendroglial tumours allowing us to identify and define specific histological characteristics for those that have lost chromosome 1p and 19q and that are known to have a preferable response to chemotherapy (Godfraind et al., in preparation).

PRIMARY RESEARCH PAPERS

1. Irrthum A, Karkkainen MJ, Devriendt K, Alitalo K and Vikkula M. Congenital hereditary lymphedema caused by a mutation that inactivates VEGFR3 tyrosine kinase.
Am J Hum Genet, 67 : 295-301, 2000.

2. Eerola I, Plate KH, Spiegel R, Boon LM, Mulliken JB and Vikkula M. KRIT1 is mutated in hyperkeratotic cutaneous capillary-venous malformation associated with cerebral capillary malformation.
Hum Mol Genet, 9(9) : 1351-1355, 2000.

3. Brouillard P, Olsen BR and Vikkula M. High resolution physical and transcript map of the locus for venous malformations with "glomus" cells (*VMGLOM*) on chromosome 1p21-22.
Genomics, 67(1) : 96-101, 2000.

4. Baurain J-F, Colau D, van Baren N, Landry C, Martelange V, Vikkula M, Boon T, Coulie PG. High frequency of autologous anti-melanoma CTL directed against an antigen generated by a point mutation in a new helicase gene.
J Immunol, 164 : 6057-66, 2000.

5. Irrthum A, Brouillard P, Boon LM, Warman ML, Olsen BR, Mulliken JB, Enjolras O and M Vikkula. Linkage disequilibrium narrows locus for venous malformations with glomus cells (*VMGLOM*) to a single YAC of 1.48 Mbp.
Eur J Hum Genet, in press.

6. Eerola I, McIntyre BAS and Vikkula M. Identification of eight novel 5'-exons in cerebral capillary malformation gene-1 (CCM1) encoding KRIT1 **Biochim Biophys Acta - Gene Structure and Expression**, in press.

REVIEW

7. Vikkula M, Boon LM and Mulliken JB. Molecular genetics of vascular malformation, Invited Mini-Review for **Matrix Biol,** in press.

CHAPTER OF A BOOK

8. Karttunen L, Felbor U, Vikkula M, Olsen BR. Vascular Matrix and Disorders. **In: Hematopoiesis: A Developmental Approach. Editor: Leonard Zon, Oxford University press**, in press.

THESIS

9. Vikkula M. Molecular Genetics of Vascular Anomalies. Thèse d'Agrégation, Université catholique de Louvain.

HORMONES AND METABOLISM

GUY G ROUSSEAU (right)
LOUIS HUE (left)

Members : *Guy G ROUSSEAU, Louis HUE, Frédéric P LEMAIGRE, Mark H RIDER*; **Associate Member** : *Patrick JACQUEMIN*; **Assistant Members** : *Luc BERTRAND , Frédéric CLOTMAN , Ulrike KRAUSE, Didier VERTOMMEN (since September);* **Michel De Visscher Fellow** : *Delphine MEISSE*; **Postdoctoral Fellows** : *Sandrine HORMAN , Christophe PIERREUX (since October);* **Graduate Students** : *Christophe BEAULOYE, Caroline BOUZIN, Nathalie HOUARD, Vincent LANNOY (until March), Anne-Sophie MARSIN, Mojgan RASTEGAR (until February), Vinciane VANHORENBEECK (since October);* **Research Associate** : *Nicolas PLUMB (since October);* **Technicians** : *Jean-François CORNUT (since September), Martine DE CLOEDT, Serge DURVIAUX (until July), Marie-Agnès GUEUNING (Mrs De Windt) (part-time), Liliane MAISIN (Mrs Lamury) (part-time), Yannick PEIGNOIS*; **Technical Staff**: *Freddy ABRASSART (part-time*); **Secretaries** *: Thérèse DEDIER (Mrs Lambert) (part-time, until June), Vivien O'CONNOR (Mrs Ouverleaux).*

The research carried out in this unit addresses the question of hormone action. Hormones act principally in two ways. They have short-term effects, which involve allosteric control and covalent modification of regulatory enzymes in metabolic pathways. Moreover, in the long-term, they control gene expression. These aspects are currently being investigated by the groups of L. Hue and G. Rousseau. As a model system, they have cloned and studied 6-phosphofructo-2-kinase (PFK-2) /fructose-2,6-bisphosphatase (FBPase-2). This bifunctional enzyme catalyzes the synthesis and degradation of fructose 2,6-bisphosphate, a potent of stimulator of glycolysis. Work on the PFK-2/FBPase-2 protein led to the understanding of its catalytic mechanisms, allosteric regulation, three-dimensional structure and hormonal control by protein phosphorylation. It also led to the identification of new partners in the insulin signalling cascade (L. Hue and M.H. Rider). Work on the PFK-2/FBPase-2 gene led to the characterization of three promoters involved in the hormonal and tissue-specific control of transcription and to the discovery of HNF-6, which is the prototype of the new ONECUT class of transcription factors (G. Rousseau and F. Lemaigre).

1. CONTROL OF FRUCTOSE 2,6-BISPHOSPHATE

Fructose 2,6-bisphosphate, discovered in this Institute by Van Schaftingen, Hue and Hers in 1980, is the most potent stimulator of 6-phosphofructo-1-kinase (PFK-1), a key enzyme of glycolysis. It is also an inhibitor of fructose-1,6-bisphosphatase, a gluconeogenic enzyme. Fructose 2,6-bisphosphate is found in eukaryotes, including yeast and higher plants, but not in prokaryotes.

Fructose 2,6-bisphosphate is synthesized from fructose 6-phosphate and ATP by 6-phosphofructo-2-kinase (PFK-2). Its hydrolysis to fructose 6-phosphate and Pi is catalyzed by fructose-2,6-bisphosphatase (FBPase-2). These two activities are catalyzed at separate sites of a bifunctional enzyme composed of two identical subunits. Our work has focused on the PFK-2 domain, in which we identified the amino acids involved in substrate binding and catalysis. We also proposed a model of the three-dimensional structure of the PFK-2 domain, which was confirmed when the crystal structure became available (Haseman et al., Structure 4:1017-1029, 1996).

We have characterized several PFK-2/FBPase-2 isoforms in mammalian tissues. They differ in PFK-2/FBPase-2 activity ratio, kinetic properties and response to phosphorylation by protein kinases. We have cloned the corresponding mRNAs and have shown that they originate from two genes (reviewed by Rousseau & Hue, Progr. Nucleic Acid Res. Mol. Biol. 45:99-127, 1993). Gene A (65 kb) gives rise to the liver (L), muscle (M) and F (fetal)-type mRNAs from distinct promoters. These mRNAs share 13 consecutive exons (2 to 14) but differ by their 5' end. Gene B (22 kb), whose exons 3 to 14 resemble exons 2 to 13 of gene A, gives rise to five mRNAs, which differ at both ends. The heart (H) isozyme, whose activity is controlled by hormones, corresponds to one of these mRNAS. Its C-terminal end contains two sites which are phosphorylated by several protein kinases and which are not present in the other isozymes. By contrast, the N-terminal end of the liver (L) isozyme contains a classical phosphorylation site for the cyclic AMP-dependent protein kinase (PKA).

The concentration of fructose 2,6-bisphosphate changes in response to metabolites, hormones, growth factors, and oncogene activation (reviewed by Hue & Rousseau, Adv. Enzyme Regul. 33:97-110, 1993). The rapid control of PFK-2/FBPase-2 is due to allosteric effectors and phosphorylation by protein kinases. In 2000, we continued our study of the control of PFK-2 in the heart. We made a detailed study of the molecular mechanisms responsible for the activation of PFK-2 by insulin, which led us to a re-investigation of the insulin signalling pathway. We also studied the mechanisms responsible for the activation of heart PFK-2 under hypoxic conditions. We found that this phenomenon is mediated by the AMP-activated protein kinase (AMPK) and we initiated a new project to search for new targets of this protein kinase.

2. INSULIN SIGNALLING PATHWAY

Mechanism of activation of heart PFK-2 by insulin

(L. Bertrand, V. Mouton, D. Vertommen, L. Hue, M.H. Rider, in collaboration with D. Alessi and P. Cohen, Dundee)

Insulin stimulates heart glycolysis by increasing glucose transport and by activating PFK-2. This in turn leads to a rise in fructose 2,6-bisphosphate (Rider & Hue, FEBS Lett. 176:484-488, 1984), which stimulates PFK-1 and hence glycolysis. We studied the mechanism by which insulin activates PFK-2 both in vitro and in intact cells. Our in vitro phosphorylation experiments showed that recombinant heart PFK-2 is phosphorylated and activated by p70 ribosomal S6 kinase (p70s6k), p90rsk and protein kinase B (PKB), which are protein kinases of the insulin signalling cascades. Our previous studies in intact cardiomyocytes (Lefebvre *et al.*, J. Biol. Chem. 271:22289-22292, 1996) showed that p70s6k and p90rsk are not necessary for the activation of PFK-2 by insulin. Therefore, PKB could be regarded as a candidate for mediating the insulin-induced activation of heart PFK-2. However, our experiments in HEK 293 cells transfected with dominant-negative constructs of PKB and 3-phosphoinositide-dependent kinase-1 (PDK-1) suggested that the phosphorylation and activation of PFK-2 by insulin was mediated by a PDK-1-activated protein kinase other than PKB (Bertrand et al., J. Biol. Chem. 274: 30927-30933, 1999).

In 2000, we partially purified a wortmannin-sensitive and insulin-stimulated protein kinase, called WISK, that phosphorylates and activates heart PFK-2. This protein kinase could be separated from PKB, serum and glucocorticoid-regulated protein kinase, (SGK) and protein kinase Cξ (PKCξ), which are all known insulin-stimulated protein kinases. In addition, WISK contained no detectable PDK-1 activity. WISK phosphorylated preferentially Ser466 of recombinant heart PFK-2 in a time-dependent manner and doubled the Vmax of PFK-2. Treatment of WISK with PP2A reversed the effect of insulin, suggesting the involvement of an upstream activating kinase (Publication 4). WISK was further purified and separated from a protein kinase related to the p21-activated protein kinase, another substrate of PDK-1 in the insulin signalling cascade. Our current efforts to identify WISK have been hampered, because of the limited amount of material and the insufficient purity of our preparations of WISK.

Insulin signalling in rat small intestine

(M.H. Rider in collaboration with the group of J.-P. Buts)

We have studied the components of the insulin signalling pathway in normal rat enterocytes from young rats. Following insulin stimulation, the insulin receptor substrates (IRS) -1, -2 and -4 became phosphorylated on tyrosine residues and the downstream components, protein kinase B and the mitogen-activated protein kinases (MAP kinase) 1 and 2, but not the p70s6k, were activated (Publication 8). The signalling pathway leading to the induction of brush border membrane sugar hydrolases by insulin at the onset of weaning is currently under study.

Ischemia-induced inhibition of insulin signalling in the heart by intracellular acidosis

(C. Beauloye, A.-S. Marsin, L. Bertrand, U. Krause, L. Hue, in collaboration with J.-L. Vanoverschelde, UCL and F. Van Stapel, KUL, Leuven)

Glucose-insulin-potassium solutions exert beneficial effects on the ischemic heart by reducing infarct size and mortality, and improving post-ischemic left ventricular function. Insulin could be the protective component of this mixture. This was evaluated by measuring changes in activity and/or phosphorylation state of insulin signalling elements in isolated perfused rat hearts submitted to no-flow ischemia. Intracellular pH (pHi) was measured by NMR techniques. No-flow ischemia antagonized insulin signalling including insulin receptor, insulin receptor substrate-1, phosphatidyl inositol 3-kinase (PI3K), PKB, p70s6k and glycogen synthase kinase-3. These changes were concomitant with intracellular acidosis. Perfusing hearts with ouabain and amiloride in normoxic conditions decreased pHi and insulin signalling, whereas perfusing at pH 8.2 counteracted the drop in pHi and the inhibition of insulin signalling by ischemia. Incubation of cardiomyocytes in normoxic conditions, but at pH values lower than 6.75, mimicked the effect of ischemia and also inhibited the insulin-stimulated glucose uptake. Finally, the *in vivo* insulin receptor tyrosine kinase activity was progressively inhibited at pH values below physiological pHi. Therefore, ischemic acidosis decreases the kinase activity and tyrosine phosphorylation of the insulin receptor, thereby preventing activation of the downstream components of the signalling pathway. We conclude that severe ischemia inhibits insulin signalling by decreasing pHi (Publication 1).

Control of insulin sensitivity by SHIP2, a lipid phosphatase

(U. Krause, L.Hue, in collaboration with S. Schurmans and C. Erneux, ULB)

Impairment of insulin action and/or secretion has a critical role in the pathogenesis of diabetes mellitus. Type II SH2-domain-containing inositol 5-phosphatase, or 'SHIP2', is a member of the inositol polyphosphate 5-phosphatase family. *In vitro* studies have shown that SHIP2 is linked to signalling events mediated by PI3K and the MAP-kinase pathway in response to insulin stimulation. In 2000, we studied mice lacking the SHIP2 gene, in collaboration with S. Schurmans and C. Erneux (ULB). Loss of SHIP2 led to increased sensitivity to insulin, which was characterized by severe neonatal hypoglycemia, deregulated expression of the genes involved in gluconeogenesis, and perinatal death. Adult mice that are heterozygous for the SHIP2 mutation had increased glucose tolerance and insulin sensitivity associated with an increased recruitment of the GLUT4 glucose transporter and increased glycogen synthesis in skeletal muscles. Our results show that SHIP2 is a potent negative regulator of insulin signalling and insulin sensitivity *in vivo* (Publication 2).

3. AMP-ACTIVATED PROTEIN KINASE

AMPK is a protein kinase that is well conserved and present in yeast, plants as well as mammalian cells. It acts as a 'fuel gauge' and is activated by an increase in the AMP/ATP ratio, i.e. when the energy content of the cell decreases. In certain cells, this protein kinase can be activated by incubation with AICA riboside, which enters these cells where it can be phosphorylated into ZMP, an analogue of AMP. AMPK switches off energy-consuming pathways, whereas it activates energy-producing pathways, such as glycolysis in the heart (see below). It also mediates the effect of nutrients on metabolism and gene expression.

Mechanism of activation of heart PFK-2 by ischemia

(A.-S. Marsin, L. Bertrand, C. Beauloye, M.H. Rider, L. Hue, in collaboration with G. Van den Berghe and M.-F. Vincent, ICP, D. Carling, London, D.G. Hardie, Dundee and P. Ferré, Paris).

In 2000, we studied the mechanism responsible for the activation of heart PFK-2 by ischemia. This phenomenon participates in the Pasteur effect, namely the stimulation of glycolysis under anaerobic conditions, and could result from protein phosphorylation. Our hypothesis was that AMPK could mediate this effect. Heart PFK-2 was phosphorylated on Ser466 and activated by AMPK *in vitro*. In perfused rat hearts, anaerobic conditions or inhibitors of oxidative phosphorylation (oligomycin or antimycin) induced AMPK activation, which correlated with PFK-2 activation and with an increase in fructose 2,6-bisphosphate concentration. Moreover, in cultured cells transfected with heart PFK-2, oligomycin treatment resulted in a parallel activation of endogenous AMPK and PFK-2. In these cells, the activation of PFK-2 was due to the phosphorylation of Ser466. A dominant-negative construct of AMPK abolished the activation of endogenous and cotransfected AMPK, and prevented both the activation and phosphorylation of transfected PFK-2

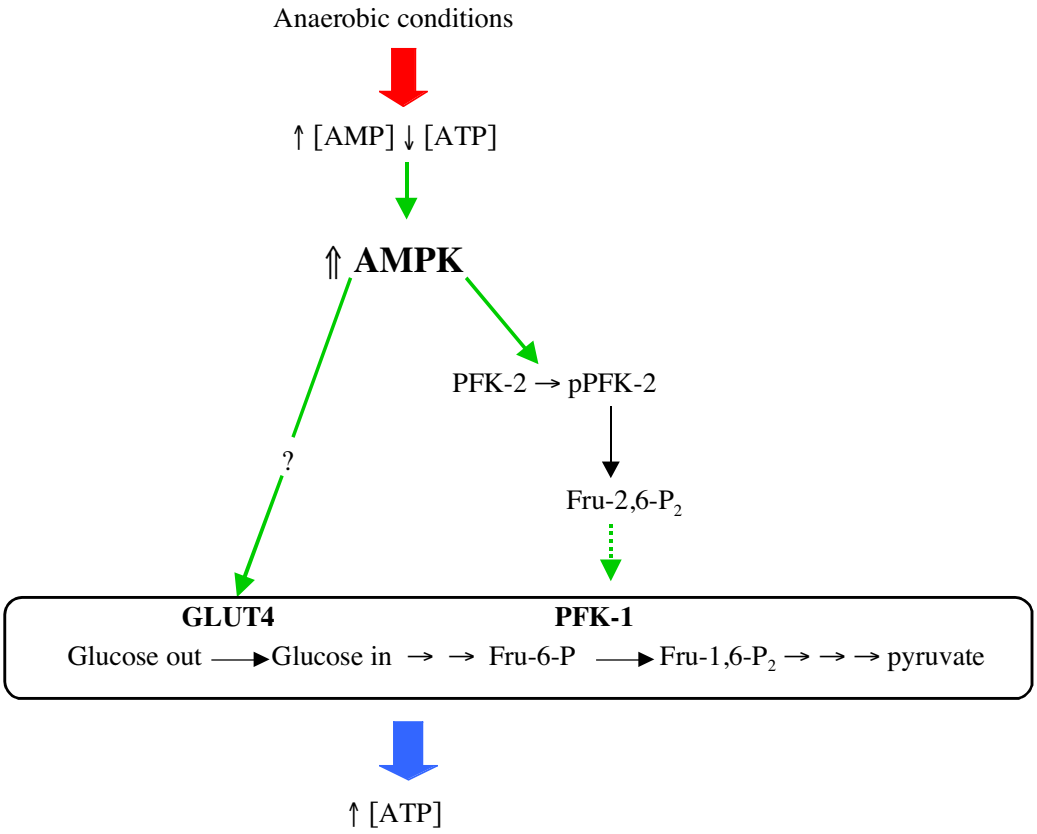

Figure 1. Mechanism of stimulation of heart glycolysis by AMPK under anaerobic conditions.
pPFK-2, phosphorylated form of heart PFK-2; GLUT4, glucose transporter. The question mark indicates an indirect and still unknown mechanism.

by oligomycin. We conclude that AMPK phosphorylates and activates heart PFK-2 *in vitro* and in intact cells (Publication 9). This AMP-mediated PFK-2 activation is likely to be involved in the stimulation of heart glycolysis during ischemia (Fig. 1)

Search for new targets of AMPK

(S. Horman, D. Meisse, L. Bertrand, A.-S. Marsin, C. Beauloye, D. Vertommen, M.H. Rider, L. Hue, in collaboration with A. Lavoinne (Rouen), P. Ferré and F. Foufelle (Paris) and M. Van de Casteele (VUB, Brussels)

Incubation of isolated hepatocytes under hypoxic conditions is known to inhibit protein synthesis. This inhibition can be mimicked by incubation with AICA riboside, suggesting that AMPK might be involved in the inhibition. In 2000, we confirmed this phenomenon and demonstrated that transfection of a dominant-negative construct of AMPK in cultured Human Embryonic Kidney (HEK) cells partially relieved this inhibition of protein synthesis. We are currently trying to identify new targets of AMPK among the factors that are known to control protein synthesis. We also observed that overexpression of a constitutively active form of AMPK in HEK cells resulted in a loss of more than 50% of the cells within three days and that incubation of hepatoma cells (FTO2B) with AICA riboside induced apoptosis of these cells as demonstrated by several methods. The underlying mechanism is currently under study.

4. REGULATION OF LIVER CARBOHYDRATE AND LIPID METABOLISM BY CELL VOLUME

(U. Krause, C. Freund, M.H. Rider, L. Hue)

In isolated hepatocytes, physiological concentrations of glutamine stimulate glycogen synthesis (Biochem. J. 248:429-437, 1987). The mechanism of this stimulation is mediated by an increase in cell volume resulting from the Na^+-dependent uptake of this amino acid. The same phenomenon was observed with those amino acids, whose transport is Na^+-dependent, and in hepatocytes incubated under hypotonic conditions (J. Biol. Chem. 265:955-959, 1990). We also showed that glutamine and other amino acids stimulate lipogenesis and inhibit ketogenesis and we concluded that swelling induces an anabolic response in the liver. The stimulation of

glycogen synthesis and lipogenesis results from an activation of glycogen synthase and acetyl-CoA carboxylase, which are key enzymes in the glycogenic and lipogenic pathways, respectively. The activity of these enzymes is controlled by phosphorylation/dephosphorylation. Both enzymes are phosphorylated at several sites by different protein kinases and, for both, the active form is the dephosphorylated enzyme.

We studied the protein phosphatases and protein kinases involved in this swelling-induced activation of glycogen synthase and acetyl-CoA carboxylase. We focused our attention on the protein kinases known to be part of the insulin signalling pathway, namely MAP-kinase 2, $p70^{s6k}$, PI3K and PKB. We demonstrated that MAP-kinase 2 and $p70^{s6k}$ are not involved, whereas PI3K is involved (Krause et al., J. Biol. Chem. 271:16668-73, 1996). In 2000, we continued our analysis of the signalling pathways and we found that the effects of insulin and swelling are mediated by different mechanisms. Insulin activates both PI3K and PKB, but does not activate $p70^{s6k}$, whereas swelling activates both PI3K and $p70^{s6k}$ but does not affect PKB. Our attempts to understand this difference have so far been unsuccessful. The difference in signalling was also confirmed by the fact that the anabolic response of the liver to glutamine-induced swelling persisted in hepatocytes from diabetic rats.

3. ROLE OF HNF-6 IN THE HORMONAL AND DEVELOPMENTAL CONTROL OF GENE TRANSCRIPTION

In 2000, we have pursued our work on hepatocyte nuclear factor (HNF)-6, a transcription factor that we discovered (Lemaigre et al., Proc. Natl. Acad. Sci. USA. 93:9460-9464, 1996) as an activator of the liver promoter of the PFK-2 gene. We have dissected the HNF-6 protein to further characterize its fonctional domains. We have also studied how the *hnf6* gene is controlled by growth hormone. To understand the role of HNF-6 in development we inactivated the *hnf6* gene in the mouse and investigated the phenotype of the *hnf6* knock-out mice.

Mechanism of action of HNF-6 on gene transcription

(V. Lannoy, A. Rodolosse, C. Pierreux, F. Lemaigre, G. Rousseau)

Many transcription factors contain a DNA-binding-region called homeodomain. The homeodomain proteins are evolutionarily conserved and play an important role in cell differentiation and in morphogenesis. Several homeodomain proteins contain a second type of DNA-binding domain. In the case of cut-homeoproteins, this second domain is called cut because it was initially described in the Drosophila Cut protein.

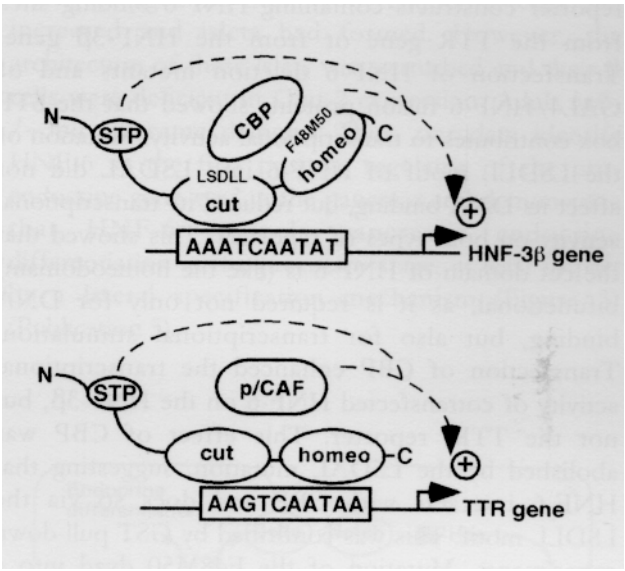

Figure 2. Mechanisms of action of HNF-6 on gene transcription. *Different HNF-6 target sequences determine distinct mechanisms for the DNA binding and activity of HNF-6 (open ovals) and impose the recruitment of different co-activators (grey ovals).*

We found that HNF-6 is the prototype of a new class of cut-homeoproteins also present in C. elegans and drosophila. We named this class ONECUT (Lannoy et al., J. Biol. Chem. 273:13552-62, 1998) because its members have a single cut domain (other cut-homeoproteins contain two or three cut domains) and a divergent homeodomain. This new type of homeodomain is characterized by a phenylalanine at position 48 and a methionine at position 50. These two residues are crucial for recognition of target genes through interaction of the third helix of the homeodomain with the major groove of DNA. We showed earlier that the homeodomain of HNF-6 is bifunctional. It is involved either in DNA binding to some targets (e.g. the HNF-6 binding site in the TTR promoter) or in transactivation of other targets (e.g. the HNF-6 binding site in the HNF-3 promoter). In this case, transactivation by HNF-6 involves the F48M50 dyad of the homeodomain. As to the cut domain, it is required for DNA binding to all the targets. The cut domain contains a LSDLL sequence,

conserved in all the ONECUT proteins, which fits with a helical motif involved in interactions of transcription factors with coactivators such as CREB-binding protein (CBP). Upstream of the cut domain, all the ONECUT proteins contain a 24 residue-long region rich in serine, threonine and proline (STP box).

To investigate further the mechanisms by which HNF-6 acts on gene promoters, we transfected cells with expression vectors for HNF-6 and luciferase reporter constructs containing HNF-6 binding sites from the TTR gene or from the HNF-3β gene. Transfection of HNF-6 deletion mutants and of GAL4/HNF-6 fusion proteins showed that the STP box contributes to transcriptional activity. Mutation of the LSDLL motif of HNF-6 into LSDAL did not affect its DNA binding, but reduced its transcriptional activity on both types of reporters. This showed that the cut domain of HNF-6 is (like the homeodomain) bifunctional, as it is required not only for DNA binding, but also for transcriptional stimulation. Transfection of CBP enhanced the transcriptional activity of cotransfected HNF-6 on the HNF-3β, but not the TTR, reporter. This effect of CBP was abolished by the LSDAL mutation, suggesting that HNF-6 interacts with CBP and does so via the LSDLL motif. This was confirmed by GST pull-down experiments. Mutation of the F48M50 dyad into a dyad found in classical homeodomains (W48H50) affected neither DNA binding nor transactivation of the TTR reporter. However, it reduced transactivation of the HNF-3β reporter and abolished the effect of CBP, as well as the binding of HNF-6 to CBP in GST pull-down experiments. Thus, on a HNF-3β target, CBP is a coactivator of HNF-6 and this involves the LSDLL motif of the cut domain and the F48M50 dyad of the homeodomain. Similar experiments with the TTR reporter showed that, in this case, p300/CBP-associated factor (p/CAF) rather than CBP acts as a coactivator and that the LSDLL motif is not involved.

We conclude from this work that HNF-6 displays two modes of action, depending on the type of target sequence (Figure 2). On targets of the TTR type, DNA binding involves both the homeodomain and the cut domain whereas transactivation involves the cut domain, the STP box and interaction with p/CAF. On targets of the HNF-3_ type, DNA binding involves only the cut domain whereas transactivation involves the cut domain, the homeodomain, the STP box and interaction with CBP (Publication 7).

Role of HNF-6 in the control of gene expression by growth hormone

(M. Rastegar, O. Lahuna, F. Lemaigre, G. Rousseau in collaboration with D. Maiter and J.P. Thissen, UCL)

We reported earlier (Lahuna et al., Proc. Natl. Acad. Sci. USA 94:12309-13, 1997) that in adult rats HNF-6 mRNA concentration in liver drops to almost undetectable levels following hypophysectomy and returns to normal after one week of growth hormone (GH) treatment. We have now shown that this results from a rapid effect of GH and we have characterized its molecular mechanism. In hypophysectomized rats, HNF-6 mRNA increased within 1 h after a single injection of GH. The same GH-dependent induction was reproduced on isolated hepatocytes. To determine whether GH regulates *hnf6* expression at the gene level, we studied its promoter. DNA binding experiments showed that (i) the transcription factors STAT5 and HNF-4 bind to sites located around -110 and -650, respectively; (ii) STAT5 binding is induced and HNF-4 binding affinity is increased in liver within 1 h after GH injection to hypophysectomized rats. Using transfection experiments and site-directed mutagenesis, we found that STAT5 and HNF-4 stimulated transcription of an *hnf6* gene promoter-reporter construct. Furthermore, GH stimulated transcription of this construct in cells that express GH receptors. Consistent with our earlier finding that HNF-6 stimulates the *hnf4* and *hnf3β* gene promoters (Landry et al., Dev. Biol. 192:247-57, 1997), GH treatment of hypophysectomized rats increased the liver concentration of HNF-4 and HNF-3β mRNAs.

HNF-6, HNF-3β and HNF-4 belong to a network which also includes CCAAT/Enhancer binding protein (C/EBP)α, the prototype of another family of liver-enriched transcription factors called bZIP. We therefore investigated the possibility that C/EBPα could mediate the effects of GH on gene transcription. In DNA-protein interactions studies we found that C/EBPα binds to the *hnf6* gene promoter. We therefore investigated whether the activity of C/EBPα was GH-sensitive and, if so, whether C/EBPα could, by controlling the *hnf6* gene, participate in the hormonal modulation of the hepatic network of transcription factors. GH treatment of hypophysectomized rats led to a rapid, transient, decrease in the amount of liver C/EBPα protein, at a time when liver HNF-6 mRNA concentration increased in response to GH. This was followed by an increase in the amount of C/EBPα mRNA and protein, concomitant with the decrease in HNF-6 mRNA. Consistent with this, transfection experiments showed that C/EBPα represses the activity of the *hnf6* gene promoter. This inhibitory effect involved a N-terminal subdomain of C/EBPα

and binding of C/EBP_ to two sites in the *hnf6* gene promoter. Together, these data demonstrate that GH stimulates transcription of the *hnf6* gene by a mechanism that involves induction of STAT5 binding and increased HNF-4 binding to the hnf6 promoter, as well as lifting of the repression exerted on that gene by C/EBP _. We conclude that HNF-6 participates not only as an effector, but also as a target, to the regulatory network of liver transcription factors, and that several members of this network are GH-regulated (Publications 6, 10, 13 and 14).

HNF-6 controls expression of the proendocrine gene ngn3 and the differentiation of pancreatic endocrine cells

(P. Jacquemin, S. Durviaux, G. Rousseau, F. Lemaigre in collaboration with M. Dewerchin, P. Carmeliet and D. Collen, Leuven, J. Jensen and O. Madsen, Copenhagen, C. Godfraind, UCL, and G. Gradwohl and F. Guillemot, Strasbourg)

The pancreas contains exocrine cells that produce digestive enzymes, ducts through which these enzymes transit to the gut, and endocrine cells that produce insulin, glucagon, somatostatin or pancreatic polypeptide. How all these types of cells derive from the same epithelium specified from the gut endoderm is unknown. One possibility is via a Notch-dependent lateral specification mechanism first identified in the nervous system. According to this mechanism, a progenitor differentiates into a given cell type thanks to a transcription factor called neurogenin-3 (NGN), but suppresses this program in a neighbouring cell by repressing its *ngn3* gene. To do so, the progenitor produces a ligand, such as Delta, which through activation of the Notch receptor of the neighbor represses ngn-3 expression. Work by others showed that in the pancreas of transgenic mice overexpressing *ngn3*, the endocrine differentiation pathway prevails over the exocrine pathway. Moreover, *ngn3*$^{-/-}$ mice lack endocrine cells.

These data made NGN3 the most upstream trancription factor for pancreatic endocrine differentiation. They also raised the question of the identity of the factor(s) that stimulate(s) the expression of *ngn3* in the pancreas. Our data suggest that this factor is HNF-6. Indeed, we found that during mouse development, HNF-6 is expressed at the onset of pancreas formation, in the undifferentiated epithelial cells that are precursors of the exocrine and endocrine pancreatic cells. Moreover, *ngn3* is expressed in cells that contain HNF-6. We have investigated the role of HNF-6 in pancreas differentiation by inactivating its gene in the mouse. In *hnf6*-/- embryos, the exocrine acini were histologically normal but endocrine cell differentiation was impaired. The expression of *ngn3* was almost abolished. Consistent with this, we demonstrated that HNF-6 binds to and stimulates the *ngn3* gene promoter. In newborn *hnf-6*-/- mice only a few endocrine cells were found and no islets of Langerhans were detected. Cells that expressed markers of undifferentiated pancreatic cells and that delineated cystic structures were also observed. At 5 weeks of age, the number of endocrine cells had increased and islets had formed. However, the_ architecture of these islets was perturbed and their cells were deficient in Glut-2 expression. Adult *hnf6*-/- mice became diabetic. Thus, our data identify HNF-6 as the first positive regulator of the pro-endocrine gene *ngn3* in the pancreas and demonstrate that HNF-6 controls pancreatic endocrine differentiation at the precursor stage, at least in part by a lateral specification mechanism (Figure 3) (Publication 5).

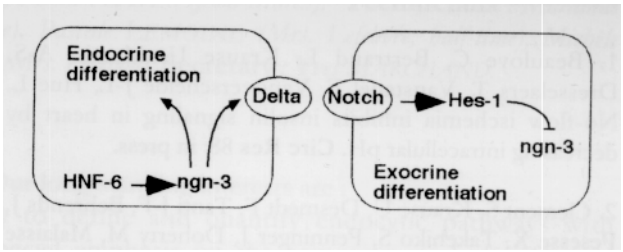

Figure 3. HNF-6 controls pancreas development by a lateral specification mechanism. *HNF-6 stimulates endocrine differenciation in developing pancreas by inducing the expression of neurogenin-3 (see text for details).*

6. PROTEIN ANALYSIS BY EDMAN SEQUENCING AND MASS SPECTROMETRY

(M.H. Rider, D. Vertommen in collaboration with the groups of J. Vandenheede (KUL), E. Van Schaftingen (UCL), F. Opperdoes (UCL)

We have used nanoelectrospray- and on-line capillary- electrospray ionisation mass spectrometry (ESI-MS) to identify autophosphorylation and regulatory phosphorylation sites in the novel protein kinase, protein kinase D. Site-directed mutagenesis of the phosphorylated residues to glutamate or alanine was undertaken demonstrating that this protein kinase is regulated by multi-site phosphorylation (Publication 12). ESI-MS analysis of tryptic fragments

from a purified preparation of a fructosamine-3-kinase led to its identification in expressed sequence tag (EST) databases and its subsequent cloning and heterologous expression (Publication 3) Futhermore, the mass spectometric analysis of peptides of the purified human red cell enzyme revealed that the initiator methionine residue was N-acetylated. The group of Professor Van Schaftingen went on to characterize the enzyme, whose physiological role is probably to initiate a pathway for the deglycation of proteins. It is known that glycated proteins play a role in the development of diabetic complications.

Edman sequencing of tryptic peptides of a purified preparation of a glycosomal malate dehydrogenase confirmed the identity of the cloned and expressed protein, whose kinetic properties were then studied (Publication 11). This low specificity isozyme is the result of a number of recent gene duplications that gave rise to a family of glycosomal 2-hydroxyacid dehydrogenases.

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CELL BIOLOGY

PIERRE J. COURTOY (front left)
YVES EECKHOUT (front right)
ETIENNE MARBAIX (rear left)
MARIE-FRANCE VANDENBROUCKE (MRS VAN DEN HOVE) (rear right)

Members *: Pierre J. COURTOY, Yves EECKHOUT, Etienne MARBAIX (part-time), Marie-France VANDENBROUCKE (Mrs. van den Hove);* **Guest Investigators** *: Idesbald COLIN, Hervé EMONARD;* **Assistant Members :** *Christine GALANT (Mrs. Ferron; part-time), Patrick HENRIET, Patrick VAN DER SMISSEN;* **Hubert and Aldegonde Brenninkmeijer Postdoctoral Fellow** *: Véronique RIGOT;* **Other Postdoctoral Fellows** *: Karine CROIZET, Christine JOSENHANS (also at MIPA Unit, till April);* **Graduate Students** *: Mustapha AMYERE, Patricia CORNET (from October), Philippe de DIESBACH, Marcel METTLEN, Christine PICQUET (Mrs. Dupriez), Anna PLATEK (from March);* **Technicians** *: Marianne EPPE (Mrs. Couvreur), Thanh LAC (Mrs. Vollemaere; half-time), Pascale LEMOINE (Mrs. Lefebvre; half-time), Michèle LERUTH, Olga MEERT (Mrs. Hazaer; half-time), Francesca N'KULI (Mrs. Pyrrhon);* **Secretary** *: Yves MARCHAND.*

In its current state, the Cell Biology Unit has grown by the effective merging of three groups, one addressing the basic mechanisms and the physiopathology of endocytosis (P.J. Courtoy), one focused on the role of endocytosis in the regulation of thyroid hormone production (M.F. van den Hove), and one exploring the regulation and roles of matrix metalloproteinases, using the human endometrium as a model system of tissue remodelling (Y. Eeckhout, P.J. Courtoy and E. Marbaix).

The *original line of research* of the Cell Biology Unit is the analysis of *endocytosis*, that is the vesicular uptake of macromolecules from the extracellular medium and the concomitant entry of plasma membrane constituents incorporated in the vesicular membrane. The endocytic apparatus comprises the pericellular membrane, primary endocytic vesicles, various (intermediate) endosomes and lysosomes. It also intersects the secretory pathway at the level of the *trans*-Golgi network.

Our long-standing interests are :
(i) to define and quantify endocytic pathways with special emphasis on the comparison of specific and bulk-membrane constituents;
(ii) to address the underlying molecular machineries using controlled perturbations including dominant-positive or -negative genetic constructs; and
(iii) to unravel the relevance of endocytosis to health, disease and therapy.

Five types of primary endocytic pits and vesicles have been described, corresponding to clathrin-coated and non-clathrin-coated micropinocytic structures (Cupers *et al.*, 1994, J. Cell Biol. 127:725-735), caveolae, macropinosomes, and phagosomes (Fig. 1). Macropinocytosis and phagocytosis are generally transient features, expressed by specialised cells. The concomitant occurrence of distinct constitutive pinocytic pathways could allow cells to internalise at different rates bulk and specific membrane constituents.

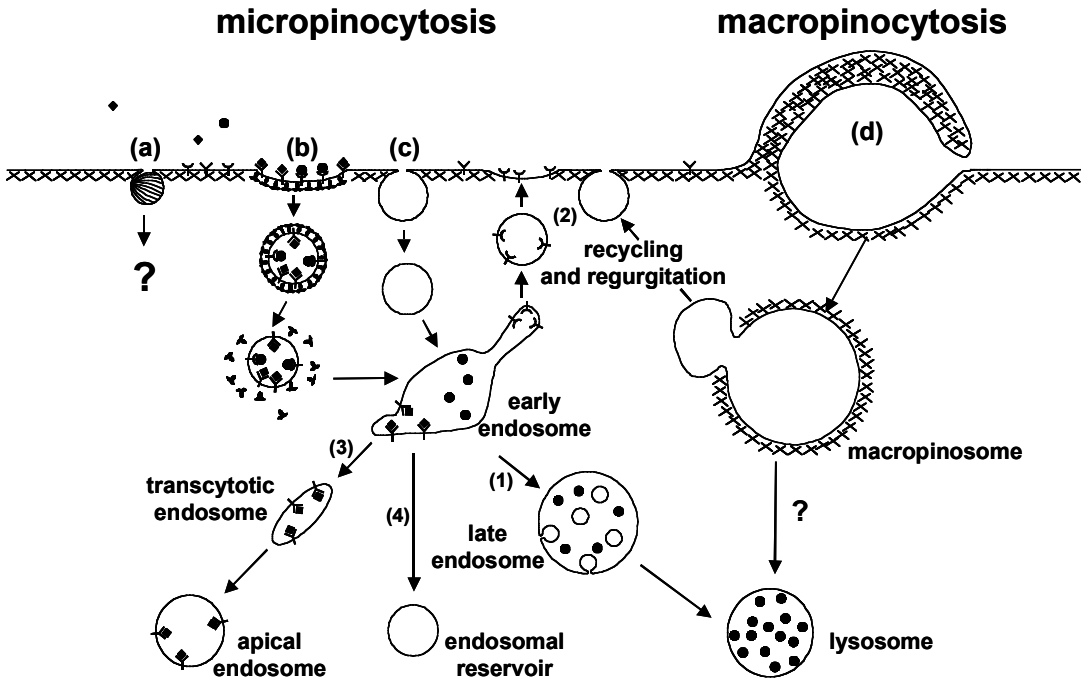

Figure 1. Pathways of endocytosis.

This scheme represents four possible modes of vesicular entry of solutes into cells : (a) caveolae; (b) clathrin- and dynamin-associated pits, or "coated pits"; (c) clathrin- and dynamin-independent micropinocytic pits; and (d) macropinocytosis. Crosses represent cortical actin. It further emphasises the endosomes as sorting organelles after micropinocytosis and outlines the four endocytic routes inside the cell : (1) the degradative pathway to lysosomes; (2) the recycling pathway, back to the plasma membrane; (3) transcytosis to the opposite membrane domain (here illustrated from baso-lateral to apical); and (4) sequestration into an endosomal reservoir. The recycling pathway may involve a TGN-stop-over.

A key feature of endosomes is their active acidification by a vacuolar H^+-ATPase. This electrogenic proton pump antagonizes its own activity, unless coupled to a chloride conductance. Based on the analysis of chloride channel-5 knock-out mice, we (Ref. 12) and others (Piwon *et al.*, 2000, Nature 408:369-373) simultaneously provided the first demonstration that a chloride channel is essential, either directly or indirectly, for the formation of primary micropinocytic vesicles.

Macropinocytosis (for a review, see Swanson and Watts, 1995, Trends Cell Biol. 5:424-428) is receiving increasing attention. First, macropinocytosis examplifies actin-driven pinocytosis. Second, macropinocytosis supports a major endocytic flow of membrane and solutes, that may satisfy nutrition requirements in *Dictyostelium* and allows for extensive "sampling" of the extracellular environment by immature dendritic cells. Third, this process resembles the "triggered, non-zipper" phagocytotic uptake of bacteria such as *Salmonella* and *Shigella*. Fourth, a relation between macropinocytosis, membrane ruffling and cell motility has emerged (Ridley, 1994, BioEssays 16:321-327). Fifth, we have shown that macropinocytosis is a constitutive feature in fibroblasts transformed by the exemplary oncogenes Src and Ras and are dissecting its signalling (Amyere *et al.*, 2000, Mol. Biol. Cell 11:3453-3467).

Endocytosis and intracellular routing of oligo-nucleotides (ODN) are not only major factors that currently limit progress in antisense therapy, but also show non-conventional properties that may illuminate new aspects of the endocytic apparatus. Having generated a probe to label *in situ* ODN-interacting partners, we have identified a membrane receptor responsible for accelerated ODN uptake (de Diesbach *et al.*, 2000, Nucl. Acids Res. 28:868-874) and are exploring its intracellular routing.

Far from being a dull final degradative compartment, lysosomes constitute a productive organelle, where signalling molecules can be generated, including antigenic peptides and thyroid hormones. However, endosomes are also competent for signal production by endocytosis. To clarify the respective contribution of endosomes and lysosomes, we are currently establishing the relationship between the progression of the hormonal precursor thyroglobulin along the endocytic apparatus and the rate of thyroid hormone production.

The *second main line of research* deals with the dynamics of *extracellular matrix breakdown*, more precisely with the mechanism of action, regulation and functions of collagenases and related matrix metalloproteinases (MMPs) in mice and man. Since the cloning and sequencing of mouse interstitial collagenase (Henriet *et al.*, 1992, FEBS Lett. 310:175-178), we have investigated the molecular properties and roles of this important mouse proteinase by expressing full-length, truncated and chimeric recombinant forms. This approach, in collaboration with the group of S. Krane (Harvard Medical School), led to the discovery of a second collagen cleavage site (Krane *et al.*, 1996, J. Biol. Chem. 271:28509-28515).

About 10 years ago, we launched the study of the regulation and functions of matrix metalloproteinases (MMPs) in human tissue remodelling, using the endometrium as a model system. The remarkable spatio-temporal relationship between the expression of MMPs and menstrual tissue breakdown *in situ* supports the hypothesis that members of this family of proteinases are responsible for the extracellular matrix breakdown that leads to menstruation. This was directly demonstrated using pharmacological inhibitors on the explant system (Marbaix *et al.*, 1996, Proc. Natl. Acad. Sci. USA 93:9120-9125). In addition, the analysis of the regulation of MMPs expression disclosed a two-level interplay, integrating an overall control by ovarian steroids and local paracrine interactions involving cytokines (Singer *et al.*, 1997, PNAS 94:10341-10345). Present studies aim at unravelling the role of these interactions to understand the cellular and molecular mechanisms leading to physiological and pathological endometrial bleeding (see Fig. 2).

Other investigations testify the CELL Unit commitment to apply basic knowledge on cellular and tissular biology, into molecular medicine and therapy. First, we pursue our collaboration with Dr. O. Devuyst and colleagues to characterize the molecular defects due to the inactivation of chloride channel-5, that may account for the various symptoms of Dent's disease (Ref. 12). Second, we are analysing the role of a NO synthase in the thyroid damage induced by cytokines, that may lead to autoimmune thyroiditis. Third, we are pursuing the investigation of the role of MMPs in pathological uterine bleeding and in the natural history of endometriotic lesions (Ref. 10).

Besides their specific projects, CELL members widely provided to local and external colleagues training in cell biology, and continuous assistance in the use of fractionation, confocal and electron microscopy equipments, as well as in data analysis. When assistance was crucial, this was reflected in coauthorships (Ref. 3, 7, 11).

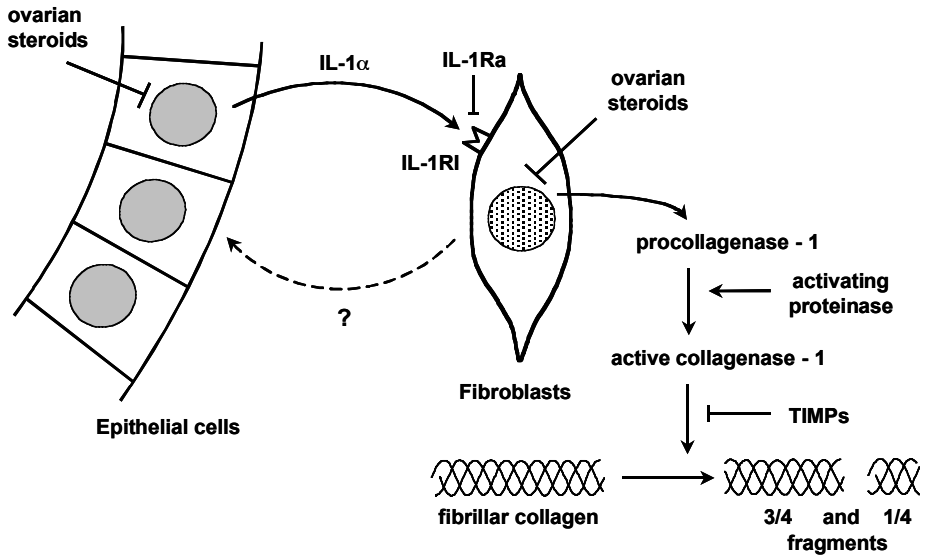

Figure 2. Model of MMP regulation in the human endometrium.
Upon ovarian steroids withdrawal, IL-1α is released by endometrial glandular cells and triggers in adjacent fibroblasts the production of proMMPs, including interstitial procollagenase-1. Fibroblast activation involves interleukin-1 receptor (IL-RI) and is opposed by various soluble factors, such as interleukin-1 receptor antagonist (IL-1Ra). Expression of proMMPs by stimulated fibroblasts is also blocked by ovarian steroids. Secreted proMMPs are then activated by proteinases currently under investigation. Active collagenase-1, if not neutralised by its tissue inhibitors (TIMPs), cleaves fibrillar collagens at position 3/4 of the distance from the amino-terminus.

1. ENDOCYTOSIS

(Group leaders : P.J. Courtoy and M.F. van den Hove)

Role of chloride channels in endocytosis : exploitation of a CLC-5 knock-out mice.

(P.J. Courtoy, in collaboration with O. Devuyst, NEFR)

One hallmark of Dent's disease, an X-linked autosomal disease due to inactivation of chloride channel-5 (CLC-5), is low-molecular weight proteinuria. This feature has been reproduced in a CLC-5 knock-out mice (Ref. 12). We postulated that proteinuria reflects a defect in receptor-mediated endocytic recapture by proximal tubular cells and tested this hypothesis by tracking the fate of injected horseradish peroxidase (a tracer of 40 kDa). Whereas this tracer was avidly taken up by proximal tubular cells of control mice, its endocytosis was almost abrogated in CLC-5 knock-out mice (Fig. 3), thereby demonstrating an essential role of CLC-5 in endocytosis of low-molecular weight proteins. Thus, CLC-5 knock-out mice are an interesting model to study the role of ionic permeabilities in endocytosis, since the deficit of the kidney-specific CLC-5 is not compensated by other chloride channels. To account for the arrest of apical endocytosis at the level of the formation of the primary endocytic vesicles, we propose that early endocytic catalysts remain trapped in distal structures and fail to be recycled to the plasma membrane, when endosomal acidification is defective.

Dynamics and regulation of oncogene-induced remodelling of cortical actin underlying constitutive macropinocytosis

(M. Amyere, M. Mettlen, A. Platek, P. Van Der Smissen and P.J. Courtoy)

Macropinocytosis results from the closure of membrane ruffles reflecting cortical actin dynamics. Rat-1 fibroblasts transformed by v-Src and by K-Ras or stably transfected for expression of (dominant-positive) wild-type phosphoinositide 3-kinase (PI3K) regulatory subunit p85 _ constitutively showed stress fiber disruption, cortical actin recruitment, ruffling and formation of macropinosomes. These alterations correlated with activation of PI3K and phospholipase C (PLC), as assayed by 3-phospho-inositides synthesis *in situ* and *in vitro* and inositol 1,4,5 trisphosphate steady-state levels, respectively; they were abolished after stable transfection of v-Src-transformed cells for truncated (dominant-negative) p85 _ expression and by pharmacological inhibitors of PI3K and PLC, indicating a requirement for both enzymes. Whereas PI3K activation resisted PLC inhibition, PLC activation was abolished by a PI3K inhibitor and dominant-negative P13K transfection, placing PLC downstream of PI3K. Thus, permanent sequential activation of both PI3K and PLC is necessary for the dramatic reorganization of actin cytoskeleton in oncogene-transformed fibroblasts, resulting in constitutive ruffling and macropinocytosis (Ref. 1).

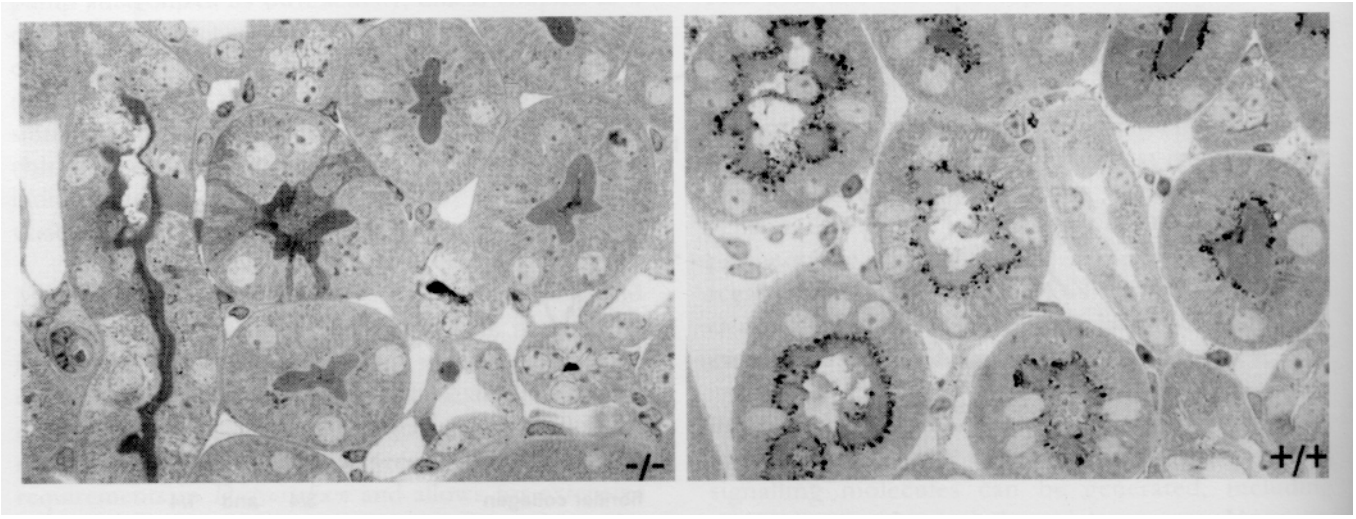

Figure 3. Deficit of a chloride channel abrogates endocytosis.
Control (+/Y) and CLC-5 knock-out mice (-/Y) were injected with horseradish peroxidase and their kidneys were processed for peroxidase cytochemistry at 5 min after injection. Notice the abundance of labelled apical endosomes in all proximal tubular cells of the control mice, at left, and their absence in mutant mice, at right (reproduced from Ref. 12).

Since most cancers are of epithelial origin, we currently extend these observations to the epithelial cell line MDCK bearing a thermosensitive v-Src kinase. In this cell line, Src reactivation also accelerates fluid-phase endocytosis. In view of the considerable interest in difference between molecular machineries underlying apical and basolateral endocytosis, we currently examine which one is affected by Src. In parallel, we are testing whether macropinocytosis, membrane ruffling and cell motility strictly correlate with one another and depend on the same signalling pathways

Role of dynamin in late endosome dynamics and trafficking of the cation-independent mannose 6-phosphate receptor

[P.J. Courtoy and L. Hilout, in collaboration with B. van Deurs (Copenhagen) and K. Sandvig (Oslo) and their colleagues]

It is well established that dynamin is involved in clathrin-dependent endocytosis, but little is known about its possible intracellular functions. Using confocal imaging, endogenous dynamin was found to be associated with the plasma membrane, the *trans*-Golgi network, and perinuclear structures containing the cation-independent mannose 6-phosphate receptor (CI-MPR). By immuno-electron microscopy, these structures were identified as late endosomes, where endogenous dynamin was preferentially localized to tubulo-vesicular appendices. Upon induction of the dominant-negative K44A dynamin mutant, endosomal tubules containing CI-MPR were formed and the bulk of CI-MPR was redistributed towards lysosomes. These results are in contrast to recent reports that dynamin is exclusively associated with endocytic structures at the plasma membrane. They suggest instead that endogenous dynamin also plays an important role in the molecular machinery supporting the recycling of the CI-MPR from endosomes to the *trans*-Golgi network. We speculate that dynamin is required for the final scission of vesicles budding from endosome tubules (Ref. 9).

Receptor-mediated endocytosis of antisense oligonucleotides

(P. de Diesbach and P.J. Courtoy).

Antisense oligodeoxynucleotides (ODN) are useful tools in experimental biology and represent a novel generic therapeutic approach. However, their potential is hampered by limited cellular uptake and transmembrane translocation to the cytosol/nucleosol where ODN targets reside. A membrane receptor for ODN has been identified in HepG2 cells by *in situ* photolabelling with a ^{125}I-ODN-benzophenone conjugate, or by ligand blotting of cell extracts with ^{125}I-ODN. Labelling was saturable and competed for by unlabelled ODN of various sequences and structures. We currently address the cellular location of this receptor by surface digestion or analytical subcellular fractionation followed by ligand blotting. Preliminary results indicate that the bulk of this receptor resides in a nonlysosomal compartment (Ref. 2).

Contribution of endosomes and lysosomes to the production of thyroid hormones

(K. Croizet, P.J. Courtoy and M.F. van den Hove)

From the comparison between various clinical and experimental conditions, we suggested that thyroid hormone production occurs not only in lysosomes as classically accepted, but also, if not predominantly, in endosomes (van den Hove *et al.*, 1998, Cell Tissue Res. 294:125-135). According to this view, endocytic trafficking of thyroglobulin is the ultimate rate-limiting factor in thyroid hormone production. To test this hypothesis, we have followed two approaches. First, by tracking the fate of internalized thyroglobulin in polarized human thyrocytes *in vitro*, we observed that thyroid hormones can be rapidly generated by endosomes and secreted in the basal medium, prior to thyroglobulin transfer into lysosomes. Second, we have compared the level of expression of the rate-limiting catalysts Rab5 and Rab7 that regulate vesicular trafficking from early and late endosomes respectively, in pairs of autonomous hyperactive adenomas and surrounding quiescent tissue from the same patient. The concentrations of Rab5 and Rab7 were found to be 2- to 3-fold higher in the hyperactive tissue than in the resting tissue. The high endocytic activity in autonomous adenomas is reflected by a decrease in soluble thyroglobulin content. A significant inverse correlation was observed between this endocytic index and Rab5 and Rab7 concentrations. Thus, the level of thyroid hormone production correlates with endocytic uptake of thyroglobulin and the expression of catalysts accelerating transfer into the most active degradative compartments (Croizet *et al.*, in preparation).

2. EXTRACELLULAR MATRIX BREAKDOWN

(Y. Eeckhout, P.J. Courtoy and E. Marbaix)

Regulation of MMP expression and activation in the human endometrium

(V. Rigot, P. Henriet, C. Picquet, P. Cornet, P.J. Courtoy, Y. Eeckhout and E. Marbaix)

Having found that both endocrine and paracrine factors control the expression and activity of the MMPs involved in the menstrual breakdown of the human endometrium, we have focused our investigations on the role of putative promenstrual (IL-1α, IL-1β, LEFTY A, TNFα) and antimenstrual cytokines (TGFβs), and on the mechanisms of proMMP activation. To determine the respective roles of these cytokines, their mRNA and protein levels are currently measured under defined hormonal conditions in (i) fresh endometrial tissue; (ii) cultured tissue explants, and (iii) primary cultures of epithelial and stromal cells. In parallel, zymographic and biochemical analyses of these samples are performed to identify the proteinases responsible for the activation of the proMMPs implicated in menstruation.

Role of extracellular matrix remodelling in cancer progression

(P. Henriet, in collaboration with Y. DeClerck and co-workers, Childrens Hospital Los Angeles, CA, USA)

It is known that the extracellular matrix regulates normal cell proliferation, and it is assumed that anchorage-independent malignant cells escape this regulatory function. We found that human M24met melanoma cells remain responsive to growth regulatory signals that result from contact with type I collagen and that the effect on proliferation depends on the physical structure of the collagen. On polymerised fibrillar collagen, M24met cells are growth-arrested at the G_1/S checkpoint and maintain high levels of $p27^{KIP1}$ mRNA and protein. In contrast, on nonfibrillar (denatured) collagen, the cells enter the cell cycle, and $p27^{KIP1}$ is down-regulated. These growth regulatory effects involve contact between type I collagen and the collagen-binding integrin $\alpha_2\beta_1$, which appears restricted in the presence of fibrillar collagen. Thus, melanoma cells remain sensitive to negative growth regulatory signals originating from fibrillar collagen, and the proteolytic degradation of fibrils is a mechanism allowing tumor cells to escape these restrictive signals (Ref. 6).

3. CELL BIOLOGICAL ASPECTS OF HEALTH, DISEASE AND THERAPY

Temporal and spatial association of matrix metalloproteinases with focal endometrial breakdown and bleeding upon progestin-only contraception

(C. Galant, P. Lemoine, P. Henriet, C. Picquet, V. Rigot, Y. Eeckhout, P.J. Courtoy and E. Marbaix)

The pathogenesis of irregular endometrial bleeding, the main reason for stopping contraception with progestins only, is unknown. Based on our reappraisal of the mechanisms of menstrual bleeding, we hypothesized that matrix metalloproteinases initiate this disorder. Volunteers upon Norplant treatment provided endometrial biopsies at the start of a bleeding episode and during nonbleeding intervals. Serum concentrations of levonorgestrel and sex hormones were not appreciably affected at bleeding, pointing to a local rather than a general disorder. Focal stromal breakdown, collagen fiber lysis, and collagenase-1 (MMP-1) messenger ribonucleic acid were evidenced in most bleeding endometria, but never in the nonbleeding ones. Immunolabeling of stromelysin-1 (MMP-3) was restricted to breaking down areas and that of gelatinase A (MMP-2) was strongly increased in these zones, as compared with nonbleeding samples or intact zones of bleeding ones. Explants from bleeding endometria also abundantly released active forms of MMP-1, MMP-2, MMP-3 and MMP-9, and less tissue inhibitor of metalloproteinases-1, than nonbleeding endometria. Collagenase-1 release closely correlated with that of interleukin-1α. In contrast, N-acetyl-β-hexosaminidase and tissue inhibitor of metalloproteinase-2 were similarly released in both groups (Fig. 4). Thus, endometrial bleeding occurs together with focal stromal breakdown, collagen lysis, expression and activation of several matrix metalloproteinases, and decreased production of tissue inhibitor of metalloproteinases-1. Taken together, these observations provide the first molecular explanation for dysfunctional endometrial bleeding (Ref. 4, 8).

Mice lacking renal chloride channel CLC-5 are a model for nephrolithiasis

(P.J. Courtoy, in collaboration with O. Devuyst, NEFR)

Nephrolithiasis (kidney stones) affects 5-10 % of adults and is most commonly associated with hypercalciuria, which may be due to monogenic renal tubular disorders. One such hypercalciuric

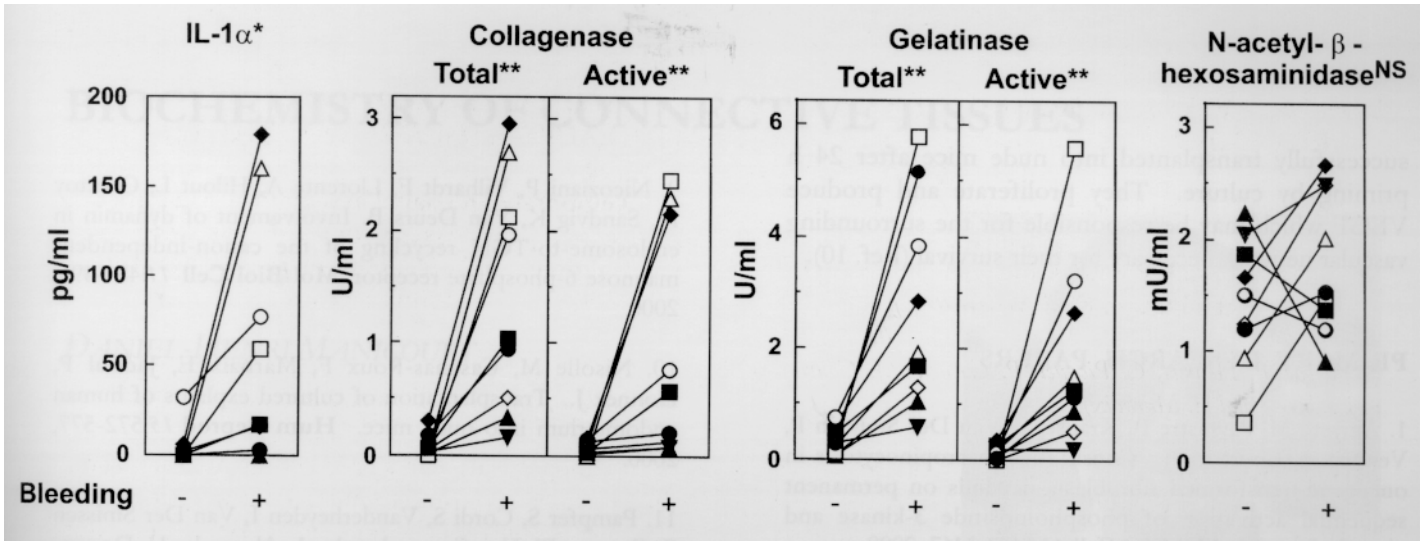

Figure 4. Dysfunctional bleeding correlates with release of interleukin-1 _, total and spontaneously active MMPs. *Paired explants from endometria sampled during bleeding (+) or between bleeding episodes (-) in the same patients were cultured for one day. Conditioned media were examined for the release of interleukin-1 _ (IL-1 _), total (APMA-activatable) and spontaneously active collagenase and gelatinase, as well as the lysosomal marker enzyme hexosaminidase for control. Bleeding significantly correlates with the release of IL-1 _ and MMPs, but not with that of the lysosomal marker (reproduced from Ref. 4). *, p<0.02; **, p<0.01; NS, not significant.*

disorder is Dent's disease, which is characterized by multiple renal proximal tubular defects including aminoaciduria and glycosuria, together with rickets in some patients. Dent's disease is due to inactivating mutations of the renal-specific voltage-gated chloride channel, CLC-5. Mice lacking CLC-5 were generated by targeted gene disruption. CLC-5-deficient mice developed renal tubular defects which included low molecular weight proteinuria (see above), aminoaciduria, and glycosuria. They also developed hyercalciuria and renal calcium deposits and eventually spine deformities. Thus, CLC-5-deficient mice provide a model for Dent's disease and may help elucidating the function of this chloride channel in renal calcium homeostasis (Ref. 12).

Involvement of nitric oxide production in cytokine-induced cytotoxic effects on thyrocytes

(I. Colin and M.F. van den Hove)

Auto-immune thyroid diseases, a significant cause of hypothyroidism in our country, result from a multistage complex process involving a large array of cytokines. We postulated that cytokine-induced effects in the thyroid gland could involve nitric oxide production, resulting into tissue destruction (Colin, 1997, Ph.D. Thesis, UCL). To test this hypothesis, we have investigated the ability of polarized human thyrocytes *in vitro* to produce $NO^{\cdot}$ in response to cytokines. We found that IL1 _, either alone or combined with IFN α triggers thyrocytes to express the inducible NO synthase (iNOS) and to secrete large amounts of $NO^{\cdot}$, resulting into LDH release. This was prevented by iNOS inhibitors, suggesting that cytokine-induced cytotoxic effects are, at least partially, mediated by $NO^{\cdot}$ and may participate in the release of auto-antigens (van den Hove *et al.,* in preparation).

A mouse model of endometriosis

(E. Marbaix, in collaboration with J. Donnez and his colleagues, GYNE)

An experimental model of endometriosis would be most welcome to better understand the molecular mechanisms underlying the natural history of this elusive yet highly prevalent disease, as well as for perspective drug studies. Biopsies of human endometrium from six patients were cultured as explants, then transplanted into nude mice. Typical endometrial glands and stroma were observed in 87% of mice 3 weeks after the transplantation. All grafts revealed histological characteristics of the follicular phase, with a high proliferative index, irrespectively of the cycle phase at the time of endometrial biopsy. An extensive vascular network developed at the interface between the graft and the surrounding tissue, and vascular endothelial growth factor (VEGF) was immunolocalized in all grafts, with a higher score in the epithelial cells than in the stromal cells. In conclusion, human endometrial explants can be

successfully transplanted into nude mice after 24 h priming by culture. They proliferate and produce VEGF which may be responsible for the surrounding vascular network necessary for their survival (Ref. 10).

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BIOCHEMISTRY OF CONNECTIVE TISSUES

DANIEL-HENRI MANICOURT

Associate Member : *Daniel-Henri MANICOURT*; **Postgraduates** *: Hafida EL AJJAJJ, Laurent BLOT (until September) ;* **Technicians** *: Anne MARCELIS, Anne VANEGEREN*

The main topics of research were as follows:

1. EFFECTS OF NONSTEROIDAL ANTI-INFLAMMATORY DRUGS ON THE OVERALL METABOLISM OF ARTICULAR CARTILAGE (Ref. 1 & 2).

Because they inhibit cyclo-oxygenase (COX), and hence the production of prostaglandins (PGs), nonsteroidal anti-inflammatory drugs (NSAIDs) are widely prescribed in patients suffering from arthritides. Two isoforms of COX have been identified thus far: COX-1, which is constitutively expressed in most tissues and COX-2, which is highly inducible in response to proinflammatory cytokines and mitogens. It is generally believed that the beneficial effects of NSAIDs are related to their ability to inhibit COX-2 whereas the gastrointestinal and renal toxicity of these drugs results from their inhibition of COX-1, a contention that has provided the basis for the development of highly selective COX-2 inhibitors. It should be however stressed that COX-1-derived PGs can contribute to the inflammatory response and that COX-2-derived PGs perform physiologically important roles such as the maintenance of normal renal function. Furthermore, COX-2-derived PGs have been implicated in the protection of the gastrointestinal tract from injury and also might have anti-inflammatory properties.

Although NSAIDs undeniably produce relief of pain and improvement of joint mobility in patients suffering from arthritides, ex vivo and in vivo studies have shown that some NSAIDs inhibit the synthesis of cartilage proteoglycans whereas others do not. This differential effect of NSAIDs on cartilage metabolism is most relevant to clinical practice since any drug, that suppresses proteoglycan synthesis and impairs the chondrocyte to repair its already damaged extracellular matrix, could potentially accelerate the breakdown of the cartilage tissue. On the other hand, although hyaluronan (HA) plays a central structural role in the supramolecular organisation of proteoglycans and, hence on the biomechanical properties of articular cartilage, the possible effects of NSAIDs on the metabolism of this glycosaminoglycan has so far focused little investigative attention.

We therefore investigated the action of celecoxib (a strong selective COX-2 inhibitor), aceclofenac and meloxicam (two moderately selective COX-2 inhibitors) on the metabolism of newly synthesised HA and proteoglycan molecules in explants from human osteoarthritic (OA) cartilage cultured in the absence and in the presence of interleukin-1 (IL-1). Results were compared with those obtained with diclofenac, a non-selective COX inhibitor which is widely used as a reference NSAID in clinical trials.

In cartilage explants with moderate and severe OA, the metabolic balance of proteoglycans and HA was unaffected by diclofenac. In contrast, and in a dose-dependent manner, aceclofenac, meloxicam and celecoxib increased the rate of synthesis of both proteoglycans and HA and also reduced significantly the net loss of newly synthesized proteoglycans and HA molecules from cartilage explants. On the other hand, when cartilage explants were cultured in the

presence of IL-1 (1 to 5 ng / ml), celecoxib, but not diclofenac, aceclofenac and meloxicam, in a dose-dependent manner strongly counteracted the down-regulation of proteoglycan and HA synthesis as well as the up-regulation of the net losses of proteoglycan and HA molecules induced by the cytokine. Further, this effect was independent of COX inhibition.

These data obtained in short-term in vitro cultures indicate that, at the concentrations found in synovial fluid, celecoxib, aceclofenac and meloxicam may exert a favourable effect on the overall metabolism of proteoglycan and HA in OA cartilage. Further, celecoxib opposes to the inhibition of cartilage matrix repair induced by IL-1 by a mechanism, which is not related to the inhibition of prostaglandin production. The elucidation of this mechanism, which might involve direct suppression of intracellular signalling and/or gene expression, is underway as it might be of great biological and therapeutic significance in arthritides.

2. MARKERS OF CONNECTIVE TISSUE METABOLISM IN HEALTH AND DISEASE.

(In collaboration with E. Thonar, Rush-Presbyterian-St Luke's Medical Center, Chicago, USA; ref. 3 & 4).

The last decade has seen the emergence of assays capable of measuring molecules whose concentrations in body fluids (joint fluid, blood and urine) provide information about specific metabolic processes occurring in vivo in joint tissues such as articular cartilage and synovium. The ability to quantify these molecules, referred to as metabolic markers, surrogate markers or simply markers, is allowing scientists and clinicians to address questions they had not been able to ask before. Although the field of research on body fluid markers of joint metabolism is still in its infancy, there already is clear evidence that the measurement of some of these markers is going to shed more light on the pathogenesis of both the preclinical and clinical stages of joint disease, including osteoarthritis, and that it will prove helpful in developing effective therapeutic treatments. For example, it is now relatively simple to rapidly determine whether a drug with documented effects on the metabolism of cultured cartilage or chondrocytes in vitro has the same effect in vivo.

The ability to measure, in small volume of serum or plasma, molecular markers that will help diagnose, monitor or prognosticate osteoarthritic changes remains a long-term goal of investigators in the field. There is good agreement that during the shorter term, one or more of these aims may be achieved by measuring a panel of a few markers. For example, observations that the quantification of some of these markers in serum may have prognostic value suggest that sensitive assays capable of measuring markers of specific metabolic processes may soon be used in the clinical assessment of patients with arthritides. In a recent review (ref 3), we have delineated the advantages as well as the limitations of measuring metabolic markers in the body fluids of patients with osteoarthritis and we have summarized advances toward the goal of providing rheumatologists with new tools for diagnosing joint changes, assessing the effects of therapy, and prognosticating disease progression.

While in vitro studies have helped improve our understanding of the mechanisms through which cytokines, growth factors, and hormones may influence cartilage metabolism, the molecular markers allow means of studying their potential effects in vivo to elucidate how they affect cartilage metabolism in disease, and to develop therapeutic interventions. Results obtained in a recent in vivo study (ref 4) strongly suggests that tumor necrosis factor alpha is involved in the up-regulation of the coordinated degradation of cartilage proteoglycans and collagen in rheumatoid arthritis, but not in osteoarthritis. Further oncostatin M is likely to act synergistically with other pro-inflammatory cytokines in up-regulating the production of metalloproteinases by chondrocytes in rheumatoid arthritis.

3. ROLE OF THE SUBCHONDRAL BONE IN THE INITIATION AND PROGRESSION OF THE OSTEOARTHRITIC DISEASE PROCESS (ref 5).

Last year, we reported that subcutaneous administration of calcitonin suppressed the responses of bone, cartilage, and synovium in the early stages of canine experimental osteoarthritis and significantly reduced the severity of the cartilage lesions (Manicourt et al. Arthritis and Rheumatism, 1999, **42**: 1159-1167). New investigations were conducted in the same animal model with a calcitonin analog, whose administration is quite easier than that of the hormone. Results clearly show that this compound reduced significantly the severity of cartilage lesions

both at the histological and biochemical levels and acted primarily by suppressing the edema and remodeling of the trabecular subchondral bone as detected by nuclear magnetic resonance imaging and bone histology (5). Obviously, subchondral trabecular bone should be a major therapeutic target in the management of osteoarthritis.

4. TOWARDS A BETTER UNDERSTANDING OF THE METABOLISM OF HYALURONAN IN CONNECTIVE TISSUES.

Research efforts are also devoted to the regulation of hyaluronan metabolism both in health and disease. In skin, which contains 50 % of total body hyaluronan, the half-life of hyaluronan is about one day, and even in as seemingly inert tissue as cartilage, hyaluronan turns over with a half-life of one to three weeks. In the blood stream, the half-life of hyaluronan is two to five minutes. All such catabolism is presumably a result of hyaluronidases. What is the nature of the control mechanisms that orchestrate such vastly different rates of turnover? The hyaluronan of vertebrate organisms can exist in many states, in a variety of sizes, in extracellular forms, free in the circulation, loosely associated with cells and tissues, tightly intercalated within proteoglycan-rich matrices such as that of cartilage, bound by receptors to cell surfaces, or even in several intracellular locations. Superimposed on these many states are the panoply of binding proteins, or hyaladherins, that decorate the hyaluronan molecule. How do mechanisms of catalysis differ among this wide range of physical and chemical states of the hyaluronan substrate? It is unlikely that hyaluronidase activity is retained in vivo in an active form within the extracellular matrix where it could cause great havoc. If it is found within the extracellular matrix, it may be in an inactive or suppressed form, perhaps bound to an inhibitor. Such a situation would parallel the relationship between the metalloproteinases and the tissue inhibitors of metalloproteinases or TIMPs that exert exquisite control over metalloproteinase activity.

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MICROBIAL PATHOGENESIS

GUY CORNELIS

Member *: Guy CORNELIS*; **Haas-Teichen Fellow** *: Simone MÜLLER;* **Van Essel Fellow** *: Sophie BLEVES (till June)*; **Societé Générale de Belgique Fellow** *: Mario FELDMAN (from September);* **Hubert and Aldegonde Brenninkmeijer Fellow***: Christine JOSENHANS (till April);* **Postdoctoral Fellows** *: Geetrui DENECKER (from February), Noureddine RAHZI (from February), Nathalie SAUVONNET (from February), Sabine TOTEMEYER (till January);* **Graduate Students** *: Rachid BENABDILLAH, Gautier DETRY, Boris FOULTIER, Nadine GROSDENT, Marie-Noëlle MARENNE, Isabelle STAINIER, Paul TROISFONTAINES;* **Technicians** *: Dominique DESNOECK, Isabelle LAMBERMONT, Claude MAUYEN, Marie-Eve RENARD (from February till November);* **Secretary** *: Marie MONTEFORTE.*

The microbial pathogenesis unit is studying the Yop virulon, a sophisticated pathogenicity system that allows bacteria from the genus *Yersinia* (*Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*) to defeat the immune system of the host. This system allows extracellular bacteria adhering at the surface of eukaryotic cells to inject bacterial effector proteins called Yops into the cytosol of these cells (Sory and Cornelis, *Mol. Microbiol.* 14:583-594, 1994), which disarms them or sabotages their communications. This system, encoded by a 70-kb plasmid, is an archetype for the so-called type III-secretion systems, encountered in more than fifteen animal and plant pathogens.

In *Yersinia*, the known effectors are YopE, YopH, YopO (called YpkA in *Y.pseudotuberculosis*), YopP (called YopJ in *Y.pseudotuberculosis*), YopM and YopT (Iriarte and Cornelis *Mol. Microbiol.* 29:915-929, 1998). YopE, YopT and YopO act on the small GTPases controlling the dynamics of actin filaments. YopE is a GTPase activating protein (GAP) that turns off the small GTPases Rac and cdc42. YopT modifies the GTPase RhoA. YopO is a actin-activated serine-threonine kinase also acting on small GTPases. YopH is an phosphotyrosine phosphatase acting on kinases of the focal adhesion. All these Yop proteins contribute to block phagocytosis. In addition, another injected Yop protein, YopP prevents the release of the proinflammatory cytokine TNFα by macrophages (Boland and Cornelis, *Infect. Immun.* 66:1878-1884, 1998) by interfering with the mitogen-activated proteins (MAP) kinases (MAPK) and NF-κB pathways. YopP also induces apoptosis of macrophages (Mills et al, *PNAS* 94:789-797,1997). Little is known about YopM, except that, unlike the other Yops, it is targeted to the nucleus.

Delivery of the Yop effectors requires the 25 YscA-Y proteins, YopB, YopD, LcrV and LcrG, which are encoded by four contiguous operons. The Ysc proteins constitute the Ysc injectisome which spans the two bacterial membranes and the peptidoglycan (Michiels et al., *Infect. Immun.* 58:2840-49,1990). YopB, YopD, LcrV and LcrG are required to transfer the effector Yops across the eukaryotic cell membrane. YopB and YopD form a pore in the animal cell membrane (Neyt and Cornelis, *Mol. Microbiol.* 33: 971-981,1999).

The proper operation of the system also requires the presence in the bacterial cytosol, of small individual chaperones, called the Syc proteins. Four such chaperones have been described so far: SycE for YopE, SycH for YopH, SycT for YopT and SycD for

YopD and YopB (Wattiau et al., *PNAS* 91: 10493-10497, 1994).

During the year 2000, we have pursued our efforts both in molecular and in cell biology. In molecular biology, we have discovered a link between the flagellum and the type III injectisome at the level of the regulation of their expression. We have investigated the role of LcrV in pore formation and we have started the analysis of a second type III secretion system that only exists in the most virulent strains of *Y.enterocolitica*. In cell biology, we focused on the two effectors YopM and YopP. We unravelled the cascade involved in the apoptosis of macrophages induced by YopP and we studied the effect of YopP on endothelial cells. We also investigated the role of all the individual Yops in the resistance towards phagocytosis by cultured J774 macrophages. Only the results that are submitted for publication are described in more detail.

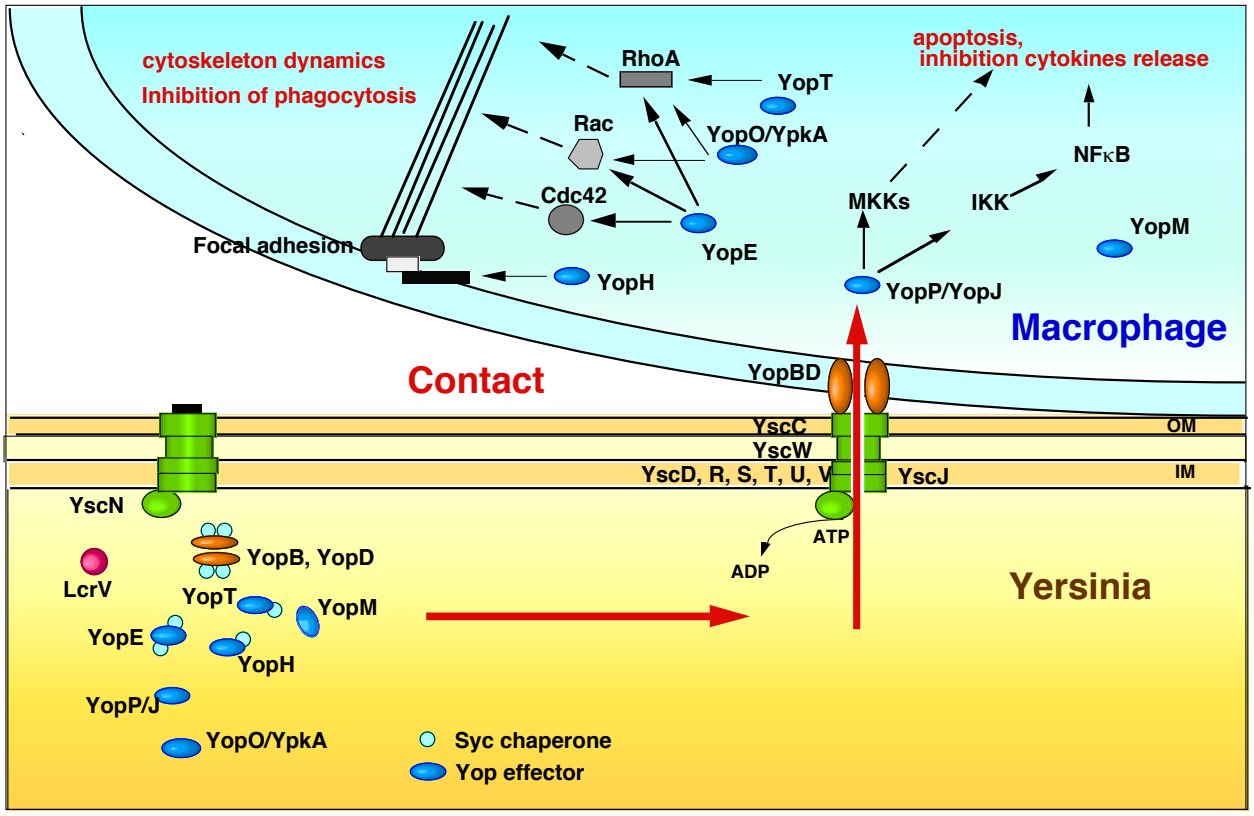

Figure 1: the basic model.

When Yersinia are placed at 37 °C in a rich environment, the Ysc injectisome is installed and a stock of Yop proteins is synthesized. During their intrabacterial stage, Yops are capped with their specific Syc chaperone, presumably to prevent premature associations. Upon contact with the eukaryotic target cell, the secretion channel opens and the Yops are exported. YopB and YopD form a pore in the target cell plasma membrane and the Yop effectors are translocated across this membrane into the eukaryotic cell's cytosol. In the eukaryotic cell, YopH dephosphorylates proteins from the focal adhesion, YopT modifies RhoA, YopE activates the GTPase activity of Rac and YopO (YpkA) phosphorylates Rac and RhoA. YopP blocks the

MAPK kinases (MKKs) and the IκB kinase β (IKK), which prevents the activation of both the MAP kinases and the NF-κB pathways, and prevents the synthesis and release of pro-inflammatory cytokines. YopP also induces apoptosis in macrophages.

1. CROSS-TALK BETWEEN THE TYPE III YOP VIRULON AND THE FLAGELLUM IN YERSINIA ENTEROCOLITICA

(Sophie Bleves, Gautier Detry and Guy R. Cornelis)

Several Ysc proteins, forming the Yops secretion apparatus, display homology with proteins of the flagellar basal body. To determine whether this relation could extend to the regulatory pathways, we analyzed the role of *flhDC*, the master regulatory operon of the flagellum, on the *yop* regulon. In a *flhDC* mutant, the Yop regulon was upregulated to the extend that Yops secretion occurred even at low temperature, a phenotype that was never observed before. The Ysc secretion machinery was thus functional at room temperature in the absence of flagella, this implying that in wild-type bacteria FlhD and/or FlhC, or the product of a FlhDC-downstream gene, represses the *yop* regulon. Transcription of *virF* and the steady-state level of this transcriptional activator were enhanced. *yop* transcription could also be detected at low temperature in the mutant. Moreover YopE was slightly more stable in the culture of *flhDC* bacteria than in a culture of WT bacteria, presumably because its secretion was improved.

2. HRPZ$_{PSPH}$ FROM THE PLANT PATHOGEN PSEUDOMONAS SYRINGAE PV. PHASEOLICOLA IS EXPORTED BY THE TYPE III SECRETION PATHWAY AND FORMS AN ION-CONDUCTING PORE IN VITRO

(Cecile Neyt and Guy R. Cornelis, in collaboration with Thorsten Nürnberger, Halle, Germany)

The *hrp* gene clusters of plant pathogenic bacteria control pathogenicity on their host plants and ability to elicit the hypersensitive reaction in resistant plants. Some *hrp* gene products constitute elements of the type III secretion system, by which effector proteins are exported and delivered into plant cells. Here we report characterization of the structure and function of *hrpZ* from the bean halo-blight pathogen, *Pseudomonas syringae* pv. *phaseolicola*. The gene was located within the *hrp* cluster and found to be transcribed independently of adjacent *hrpA*. HrpZ was secreted in a *hrp*-dependent manner in *P.s.* pv. *phaseolicola* and was also exported by the type III secretion system in the mammalian pathogen *Yersinia enterocolitica*. The protein was found to integrate stably into artificial membranes. Under symmetric ionic conditions, addition of 2nM purified recombinant HrpZ$_{Psph}$ to the *cis*-compartment of planar lipid bilayers provoked an ion current with a large unitary conductivity of 207 pS. The ion-conducting pore was permeable for monovalent cations but did not mediate fluxes of Cl^-. This pore-forming activity may allow nutrient release and/or delivery of virulence factors during bacterial colonization of host plants. (Publication 4)

3. STUDY OF THE YopP-INDUCED APOPTOSIS OF MACROPHAGES.

(Geertrui Denecker, Cecilia Geuijen, Rachid Benabdillah and Guy R. Cornelis in collaboration with Wim Declerck and Peter Vandenabeele from the University of Ghent and VIB)

The delivery of YopP into macrophages not only prevents the release of the pro-inflammatory cytokines but also triggers apoptosis. The exact mechanism of this induction remains unknown. Recently it was reported by others that YopJ, the homologue of YopP in *Y.pseudotuberculosis* is a member of an ubiquitin-like protein cysteine protease family and that the catalytic core of YopP is required for its inhibition of the MAPK and NF-κB pathways. We analyzed the YopP-induced apoptotic signal transduction pathway. YopP-mediated cell death could be inhibited by the addition of the zVAD caspase-inhibitor, but not by DEVD or YVAD. Generation of truncated Bid (tBid) was the first apoptosis-related event that we observed. The subsequent translocation of tBid to the mitochondria induced the release of cytochrome c, leading to the activation of caspase-9 and the executioner caspases-3 and-7. Inhibition of the post-mitochondrial executioner caspases-3 and -7 by the use of the DEVD caspase inhibitor did not affect Bid cleavage. Bid cleavage could not be observed in a *yopP*-deficient *Yersinia* strain, showing that this event requires YopP. Disruption of the catalytic domain of YopP abolished the generation of tBid, thereby hampering the induction of apoptosis by *Yersinia*. This finding supports the idea that YopP induces apoptosis by directly acting on cell death pathways, rather than being the mere consequence of gene-induction inhibition in combination with microbial stimulation of the macrophage.

4. EFFECT OF YERSINIA ENTEROCOLITICA YopP ON THE INFLAMMATORY RESPONSE MEDIATED BY HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

(Sabine Tötemeyer, Dominique Desnoeck and Guy R. Cornelis)

Yersinia enterocolitica proliferates in the hostile environment of the lymphatic system. This requires disarming of the host immune response. Translocated effector proteins, Yops, interfere with signalling pathways of the host cell. YopP has been shown to inhibit the activation of mitogen-activated protein kinase kinase (MKK) superfamily and IKKβ, thus blocking simoultaneously MAPK and NF-κB signalling pathways. These pathways are involved in mediating the inflammatory response. We have shown that YopP also plays a key role in subverting the host response to inflammation mediated by human endothelial cells in response to *Y. enterocolitica* infection by down regulating the expression of the cellular adhesion molecules E-selectin, ICAM-I and VCAM-I and the production and release of the pro-inflammatory cytokines IL-6 and IL-8.

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6. Cornelis GR "Type III secretion a bacterial device for close combat with cells of their eukaryotic host" **Philosophical transactions, Biological sciences (Royal Society)** Volume 355, pp. 681-693, 2000.

7. Cornelis GR "Molecular and cell biology aspects of plague". **Proceedings of the National Academy of sciences (USA)** 97, 8778-8783, 2000.

8. Bleves S and Cornelis GR. "How to survive in its host: the *Yersinia* lesson". **Microbes and Infection**, 2:1451-1460, 2000.

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10. Boland A and Cornelis GR. "Interaction of Yersinia with host cells". **In "Bacterial invasion into eukaryotic cells". "Subcellular biochemistry". Edited by J. Hacker and T. Oelschlager. Published by Plenum**, pages 343-382, 2000

11. Boyd A and Cornelis GR. "Yersinia**" In "Principles of bacterial pathogenesis" Edited by E. Groisman. Academic Press**, pages 227-264, 2000.

12. Boyd A and Cornelis GR. In "Unraveling bacterial pathogenesis: a molecular mechanistic approach". Edited by Ron Taylor. Blackwell Science Publisher. In press.

THESIS

13. Stainier I. Doctorat en Sciences Biomédicales. "The type III injectisome of Yersinia: regulatory and structural aspects". Université catholique de Louvain.

THEILER'S VIRUS

THOMAS MICHIELS

Member *: Thomas MICHIELS;* **Graduate students** *: Karima JNAOUI, Olivier VAN EYLL, Vincent VAN PESCH;* **Technicians** *: Pierre RENSONNET, Muriel MINET (from October).*

Theiler's murine encephalomyelitis virus (TMEV or Theiler's virus) is a murine picornavirus responsible for infections of the central nervous system. TMEV isolates are generally classified in two subgroups on the basis of the pathology they induce in the central nervous system of susceptible mice. The first subgroup contains two highly neurovirulent strains, GDVII and FA, that replicate permissively in brain neurons, causing an acute lethal polioencephalomyelitis. Viruses of the second TMEV subgroup, such as DA or BeAn, do not kill the mice but provoke a chronic demyelinating disease considered as a model of Multiple Sclerosis. These viruses have a striking ability to persist in the central nervous system in spite of a specific cellular and humoral immune response. Persistence of the virus is associated with a strong inflammatory response and with lesions of primary demyelination.

The genome of Theiler's virus is an 8 kb-long positive strand RNA molecule (Fig. 1). This genome is infectious and generates viral progeny after its transfection in BHK-21 cells.

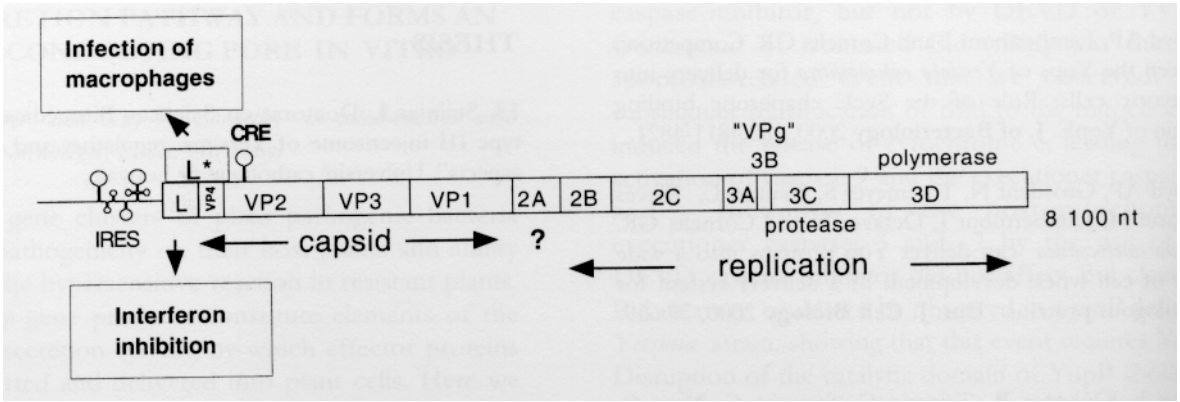

Figure 1. Genome of Theiler's virus: *A large open reading frame encodes a 2000 amino acid-long polyprotein that is cleaved, by autoproteolytic activity, into 12 mature proteins. An additional protein (L*) is encoded by an alternative open reading frame, overlapping regions L, VP4 and VP2. Translation of both ORFs is driven by an Internal Ribosome Entry Site (IRES) present in the 5' non-coding region of the genome. Protein L* was shown to facilitate the infection of macrophages. Protein L inhibits immediate-early type-I interferon (this work). The role of protein 2A is unknown. 60 copies of proteins VP1 to VP4 assemble to form the viral capsid. 3B (also termed VPg) is covalently linked to the 5' end of the RNA molecule during encapsidation and replication. 3C is the protease responsible for most of the cleavages occurring during polyprotein processing. 3D is the RNA-dependent RNA polymerase. Proteins 2B, 2C, 3A participate in the replication complex. A cis-acting replication signal has recently been mapped to the VP2 coding sequence and is denoted CRE for "cis-acting replication element".*

Theiler's virus is thus an outstanding model to analyze the basic mechanisms of viral persistence and demyelination. i) It replicates and persists in the central nervous system in the face of a specific humoral and cellular immune response. ii) it induces chronic demyelination in mice. iii) it is a natural pathogen of the mouse and allows the experimental analysis of a natural host-pathogen interaction. iv) its genome is cloned and manipulable by the tools of molecular biology.

Our work aims at understanding how a virus can persist in the central nervous system of an immunocompetent host. We have undertaken studies destined to identify the viral and cellular determinants of tropism and persistence.

1. INHIBITION OF TYPE-I INTERFERON PRODUCTION BY THE LEADER PROTEIN

(V. van Pesch, and T. Michiels)

The leader (L) protein encoded by Theiler's virus is a 76 amino acid-long peptide containing a zinc-binding motif. It was suggested that this protein might interfere with the type-I (α/β) interferon (IFN) response.

To test the involvement of the leader protein in IFN production inhibition, we compared the production of interferon by L929 cells infected with either the wild-type virus or a mutant virus carrying a mutation in the zinc finger motif of the L protein. We showed that the L protein indeed inhibits the production of type-I interferon. Furthermore, using RT-PCR, we observed that inhibition was specific for $\alpha 4$ and β interferons. These interferon subtypes correspond precisely to those that are known to be activated early in response to viral infection (immediate-early interferons).

A potential target of the leader protein is IRF-3, as this cell factor is known to specifically activate the transcription of IFN-β and IFN-$\alpha 4$. IRF-3 is constitutively present in the cytoplasm of non-infected cells. Viral infection triggers a signaling cascade which leads to the translocation of IRF-3 to the nucleus where it activates the transcription of the IFN genes. Experiments are in progress to determine whether the leader peptide interacts with IRF-3.

Mutation of the zinc-finger was sufficient to abolish the anti-IFN activity of the L protein, outlining the importance of this motif in the protein function. In the Picornavirus family, only Aphthoviruses and Cardioviruses express a leader protein. The leader protein of aphthoviruses is a protease unrelated to the L protein of TMEV. In contrast, the zinc finger is highly conserved in the leader proteins of Cardioviruses including TMEV and Encephalomyocarditis virus (EMCV), suggesting that the anti-IFN activity of the protein might be conserved in the latter virus.

In agreement with the anti-interferon role of the L protein, a Theiler's virus mutant bearing a mutation in the zinc-binding motif of L was dramatically impaired in its ability to persist in the central nervous system of SJL/J mice.

2. INFLUENCE OF THE L* PROTEIN ON MACROPHAGE INFECTION, VIRAL PERSISTENCE AND NEURO-VIRULENCE

(O. van Eyll, and T. Michiels)

In persistent strains of Theiler's virus, an 18 KDal protein called L* is encoded by an alternative open reading frame (ORF) overlapping the L-VP4-VP2 coding regions of the main ORF (see Fig. 1). In neurovirulent strains, however, the entire open reading frame is conserved but the AUG codon initiating translation of the L* ORF is replaced by an ACG codon.

The L* protein was shown to enhance the infection of macrophage cell lines and to be critical for persistence of the virus in the central nervous system of the mouse. However, the role of this protein in vivo was questioned since some chimeric and mutant viruses carrying an AUG to ACG mutation of the initiation codon were reported to persist.

We compared the phenotype of L* mutant viruses carrying either an AUG to ACG mutation at the level of the initiation codon or a stop codon mutation introduced at different locations of the L* ORF. We confirmed previous results showing that a virus carrying the AUG to ACG mutation was able to persist in the central nervous system of the mouse and we showed that the effect of L* was due to the L* protein rather than to a possible competition mechanism for the translation initiation of the two overlapping ORFs. Surprisingly, the phenotype of the stop codon mutants differed from that of the initiation codon mutants. Analysis of this phenomenon is in progress.

3. ANALYSIS OF THE INTERNAL CIS-ACTING REPLICATION SIGNAL.

(T. Michiels and P-E. Lobert,, in collaboration with N. Escriou, Pasteur Institute, Paris)

We detected previously, in the VP2-coding region of Theiler's virus, a RNA sequence acting as a signal for genome replication. This signal was predicted to consist in a stem and loop RNA structure with a conserved nucleotide sequence in the loop. The replication signal is conserved and could be functionally exchanged among viruses of the genus *Cardiovirus*. Similar replication signals were recently discovered in the coding sequences of two other picornaviruses: human rhinovirus-14 and poliovirus. Although the replication signals found in those viruses probably play a similar role, they occur at different locations of the genome (VP1 in rhinovirus-14 and 2C in poliovirus) and could not functionally replace the signal of Theiler's virus. Only the AAACA sequence was found to be conserved in the loops of the different replication signals identified so far. This sequence was proposed to serve as a template for VPg uridylation by the 3D polymerase of the virus, the initial step of viral RNA replication.

We introduced point mutations affecting either the secondary structure of the stem or the A in the (A)AACA conserved sequence of the loop (Fig. 2).

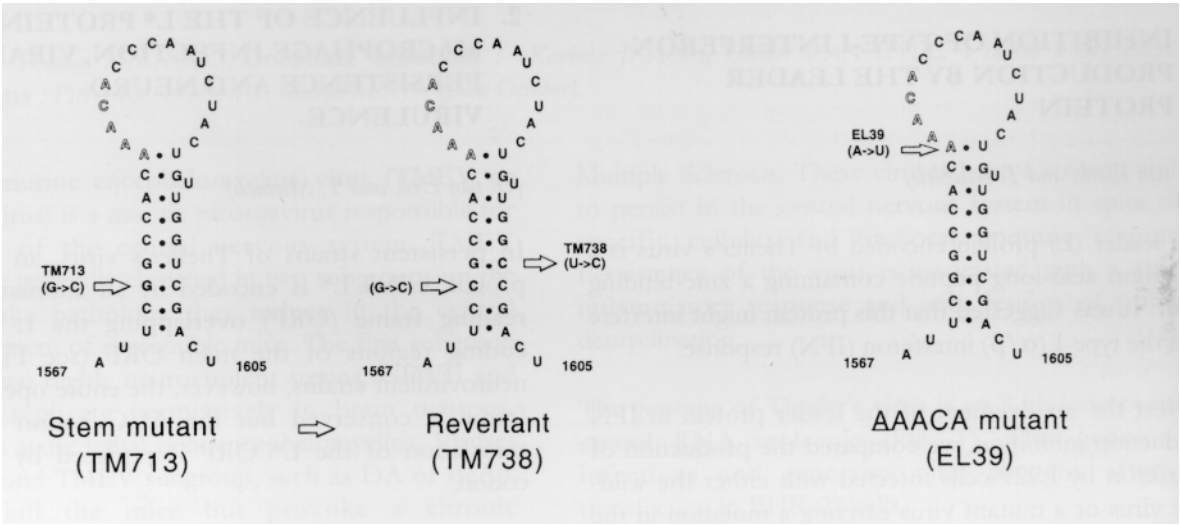

Figure 2. Analysis of the cis-acting replication signal: *A G->C mutation in the stem of the CRE (in virus TM713) could be rescued (in revertant TM738) by a compensatory mutation (U->C) occurring on the complementary strand and restoring RNA secondary structure. In contrast, all the revertants selected from the EL39 virus carrying an A->U mutation in the AAACA sequence of the loop reverted to the wild-type sequence, outlining the importance for replication, of this conserved sequence.*

We transfected the mutated genomes in BHK-21 cells and selected, by plaque assay, the emergence of revertants that regained the ability to replicate and propagate in these cells. Some revertants obtained from the stem-mutant virus carried second site mutations that restored the secondary structure of the stem on the positive strand of the RNA. These data confirm the influence of RNA secondary structure of this region and show that the replication signal acts on the positive strand RNA molecule. Interestingly, all the revertants isolated from the mutant carrying the mutation in the AAACA sequence of the loop had regained the wild-type sequence. This observation confirms the critical role of the conserved AAACA sequence.

Recent models propose that the internal replication signal is used as a template by the viral polymerase to uridylate VPg. Thus, a complex consisting of VPg, the 3D polymerase, and other viral (3CD) or cellular factors would assemble on the replication signal. After uridylation of VPg, the VPg-pU-pU/3D complex would jump to the 3' end of the positive strand RNA molecule to initiate synthesis of the negative strand.
Using a trans-complementation assay, we showed that the replication signal was required in cis, suggesting a physical coupling between the uridylation step and negative strand synthesis initiation.

4. INTERACTION OF THEILER'S VIRUS WITH THE CELL

(K. Jnaoui, and T. Michiels)

We isolated and cloned variants of the DA1 (persistent) and GDVII (neurovirulent) viruses that were adapted to grow on CHO-K1 cells. Mutations responsible for adaptation of the virus to these cells occurred in the capsid of the virus and affected amino acids contained in loops that are exposed at the surface of the virus. Such amino acids that affect the tropism of the virus in vitro most probably correspond to the residues that interact with the host receptor.

First, we showed, by infecting characterized CHO-K1 mutant cell lines, that entry of either virus strain was not dependent on the presence of glycosamineglycans at the cell surface.

Second, we isolated a series of CHO-K1 cell mutants resistant to TMEV infection. Unlike what we observed in the case of L929 cells, the mutants that we isolated were resistant to both the DA1 and the GDVII virus strains. Analysis of selected CHO-K1 cell mutants revealed that they all had defects in the protein glycosylation pathway. This indicates that both the DA and GDVII groups of strains recognize glycoproteins for entry into the cell.

Persistent strains but not neurovirulent strains of Theiler's virus were reported to bind sialic acid. Interaction with sialic acid involved protein loops exposed at the surface of the viral capsid and in particular the EF loop of the capsid protein VP2. We confirmed this interaction for the DA virus strain. In addition, we showed that some variants of the virus, including viruses derived from GDVII, could interact with other types of sialic acid through different capsid determinants. Subtle changes in the capsid had a dramatic impact on persistence of the DA virus or neurovirulence of the GDVII virus.

Thus we believe that subtle changes in the surface loops of the capsid modulate the affinity of a given capsid for the receptor present on a given cell line and thereby modulate the tropism of the virus and the pathology that it causes in the central nervous system.

PRIMARY RESEARCH PAPER

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TROPICAL DISEASES

FRED R. OPPERDOES
PAUL A.M. MICHELS

Members*: Fred R OPPERDOES, Paul AM MICHELS;* **Assistant Members***: Véronique HANNAERT, Jean-Pierre SZIKORA;* **ICP Fellow***: Emma SAAVEDRA (from March);* **Graduate Students***: Marie-Astrid ALBERT, Stéphane DE WALQUE (till March), Daniel GUERRA (from October), Hanane KRAZY, Juliette MOYERSOEN, Alain NAHUM (till October), Cédric YERNAUX (from October);* **Technicians***: Freddy ABRASSART, Nathalie CHEVALIER, Dominique COTTEM, Christian VAN LANGENHOVE, Joris VAN ROY;* **Secretary***: Françoise MYLLE.*

Trypanosomes are responsible for human sleeping sickness in tropical Africa and for a similar disease called 'nagana' in cattle. These are very serious diseases, with fatal outcome if left untreated. The presently available drugs are not very efficient and cause serious side effects. Moreover, development of drug resistant parasites is becoming a major problem. Therefore new drugs are badly needed.

Trypanosoma brucei, when it resides in the mammalian bloodstream, relies entirely on glycolysis for its ATP supply. Moreover, the parasite, like all other protozoan organisms belonging to the order Kinetoplastida, is characterized by a unique form of metabolic compartmentation; the majority of the enzymes of the glycolytic pathway is sequestered in peroxisome-like organelles called glycosomes. This compartmentation has been studied in most detail in *T. brucei*. For the above reasons the glycolytic pathway is considered a validated and promising target for new drugs to be designed. Since many years we study the kinetic and structural properties of the glycolytic enzymes of *T. brucei* and related parasites such as *Leishmania mexicana*, and use the collected information for the design of effective and parasite-enzyme selective inhibitors by structure-based and catalytic mechanism-based approaches. In addition we have extended our research with an analysis of the control of the glycolytic flux *in vivo*. The flux control is being studied in a quantitative manner by using a mathematical model prepared on the basis of the experimentally determined kinetic properties of all enzymes constituting the pathway, and by *in vivo* experiments in which the activity of different enzymes of the pathway is varied by either biochemical or genetic means. Such experiments could provide insight into the consequences of the compartmentation of the pathway and provide information as to which enzymes of the pathway are the most efficient targets for drugs. Recently our research was also extended towards the enzymes of another pathway of carbohydrate metabolism: the hexose-monophosphate pathway. Some of the enzymes of this pathway, which are involved in the generation of intermediates essential for cell growth and division and its protection against oxidative stress, were also found to be associated, in part, with the glycosomes in *T. brucei*, and this triggered our interest in their function as glycosomal proteins.

In the year 2000 the following achievements were made.

1. ENZYMES OF THE GLYCOLYTIC PATHWAY

Cloning and characterisation of genes for glycolytic enzymes, expression and characterisation of recombinant enzymes.

(V. Hannaert, I. Kralova, F. Duffieux and P. Michels)

The genes of the cytosolic *T. brucei* enzymes phosphoglycerate mutase (PGAM) and enolase (ENO) have been cloned and sequenced. Consequently, genes of all enzymes of the pathway have been characterised by our laboratory. In addition, we have cloned and sequenced the genes for 6-phosphofructo-2-kinase (PFK2) and fructose-2,6-bisphosphatase (FBPase2), two enzymes responsible for the synthesis and hydrolysis of fructose 2,6-bisphosphate, a potent allosteric effector of pyruvate kinase (PYK) and possibly the major regulator of the pathway. *T. brucei* PGAM appears to belong to a class of cofactor 2,3-bisphosphoglycerate independent mutases, whereas humans possess a cofactor-dependent enzyme. The enzymes belonging to these different classes are not homologous, rendering the trypanosome enzyme a highly promising drug target.

The PGAM and ENO have been expressed as recombinant enzymes in *Escherichia coli* and purified. Kinetic properties of both enzymes have been determined. In addition several other enzymes, of which the genes were cloned previously, and which are directly or indirectly involved in glycolysis of bloodstream-form *T. brucei*, have been over-expressed, purified and kinetically characterised: glucose-6-phosphate isomerase (PGI), phosphofructokinase (PFK), glycerol-3-phosphate dehydrogenase (GPDH) and glycerol kinase (GK). Crystallographers in Seattle (Prof. W. Hol) and Edinburgh (Dr L. Gilmore) have, in collaboration with us, solved the crystal structure of several trypanosomatid glycolytic enzymes: aldolase (ALD), GPDH and PYK. Based on these crystal structures, and those determined for other enzymes previously (glyceraldehyde-3-phosphate dehydrogenase –GAPDH- and 3-phosphoglycerate kinase –PGK-), and on insight in kinetic properties and catalytic mechanism, inhibitors have been designed and synthesised (by our colleagues Prof. J. Périé, Toulouse and Prof. M. Gelb, Seattle). Promising inhibitors, selective for parasite GAPDH and ALD, with K_i's in the nanomolar range and inhibiting growth of parasites in culture without affecting growth of cultured human cells have already been obtained.

Some proteins (hexokinase –HXK-, PFK2/FBPase2) are only very poorly expressed in *E. coli*, or are only produced in insoluble form under all growth conditions used for the bacteria (different growth media, temperature, induction regime) and with whatever *E. coli* expression system applied (different strains, different vectors, different types of fusion proteins). For the production of these recombinant proteins, we are currently employing mammalian cells and a recently developed system for large-scale *in vitro* expression of proteins (Roche 'Rapid Translation System').

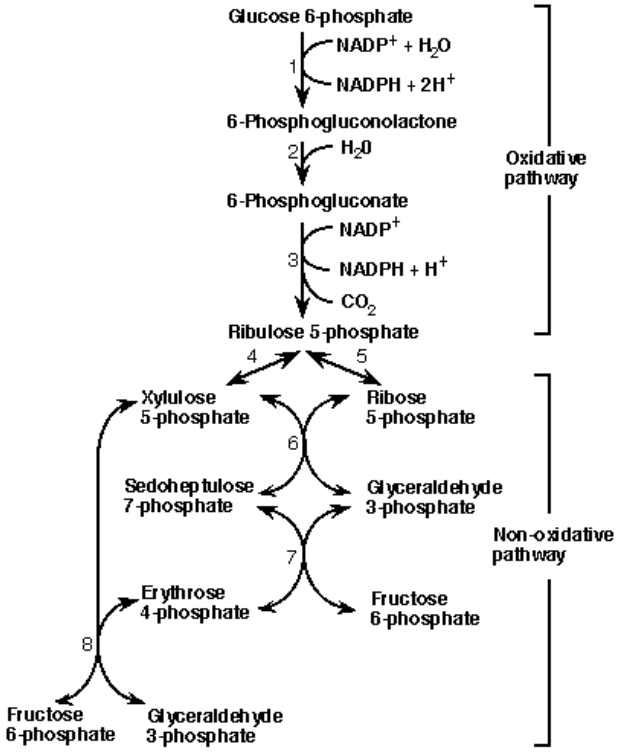

Figure 1. The Hexose-Monophosphate Pathway. Enzymes: 1, Glucose-6-phosphate dehydrogenase; 2, 6-phosphogluconolactonase; 3, 6-phosphogluconate dehydrogenase; 4, ribulose 5-phosphate 3-epimerase; 5, ribulose 5-phosphate isomerase; 6, transketolase; 7, transaldolase; 8, transketolase.

2. ENZYMES OF THE HEXOSE-MONOPHOSPHATE PATHWAY

Glucose-6-phosphate dehydrogenase and 6-phosphogluconolactonase

(F. Duffieux, E. Saavedra, P. Michels and F. Opperdoes)

Fragments of the *T. brucei* genes possibly coding for the first two enzymes of the hexose-monophosphate pathway, glucose-6-phosphate dehydrogenase

(G6PDH) and 6-phosphogluconolactonase (6PGL), were identified by *in silico* screening of DNA sequences obtained through the "Trypanosome Genome Project". The full-length genes were subsequently cloned, and sequenced. Contrary to expectation the G6PDH sequence did not reveal a peroxisome-targeting signal (PTS), while 50% of its activity is found in glycosomes. However, the lactonase, of which some 15% of the activity is glycosomal, contains a C-terminal motif showing similarity to an established type of targeting signal (PTS1), in agreement with its subcellular localisation. Subsequently, the corresponding genes from *L. mexicana* were cloned and sequenced and to our surprise here the G6PDH contained a PTS1 signal, while the 6PGL contained a putative PTS2 signal. The presence of a peroxisome-targeting signal in at least two enzymes of the hexose-monophosphate shunt in trypanosomatids confirms the importance of glycosomes in functions other than glycolysis, such as biosynthetic processes and protection against oxidative stress. The two enzymes from *T. brucei* were overexpressed in *E. coli*, purified and characterised. These studies confirmed the identity of the gene products as G6PDH and 6PGL. In collaboration with Dr. Duffieux (now at the Ecole Polytechnique, Palaiseau, France) the three-dimensional structure of the *T. brucei* lactonase is being solved using the technique of nuclear magnetic resonance.

3. ANALYSIS OF THE CONTROL OF THE GLYCOLYTIC FLUX

(M.-A. Albert and P. Michels in collaboration with B. Bakker and H. Westerhoff, Free University of Amsterdam, The Netherlands, and S. Helfert and C. Clayton, University of Heidelberg, Germany)

Previously we reported that a mathematical model of the parasite's glycolysis was developed based on the kinetic data as available for the enzymes involved. This model could predict successfully the experimentally determined fluxes and metabolite concentrations in trypanosomes incubated under aerobic and anaerobic conditions, and the intracellular concentration of glucose as a function of the extracellular concentration. The modelling proved that trypanosome glycolysis can be understood in terms of the kinetics of the glycolytic enzymes. We have now upgraded our model using the kinetic data that have become available more recently. The model was also used to determine the rate-limiting steps of trypanosome glycolysis. Under physiological conditions, most control was exerted by the plasma-membrane glucose transporter. At higher glucose concentrations, control shifted and became shared by four enzymes: ALD, GAPDH, GPDH and PGK. Only little control is exerted by HXK, PFK and PYK. By calculating which steps need the least inhibition to achieve a certain reduction of the flux, the glucose transporter and the same set of four enzymes were also identified as the most promising drug targets. The enzymes catalysing irreversible reactions, HXK, PFK and PYK, seem to possess substantial overcapacity according to the model. Furthermore, using the model, the efficacy of competitive versus noncompetitive enzyme inhibitors and of inhibitor combinations was calculated.

The flux control coefficient of the glucose transporter has been experimentally determined by titration with inhibitors. The prediction from the model was confirmed: At low glucose concentrations (0.5 mM), the transporter assumes all flux control, at the physiological concentration of 5 mM it has 40% control. At this moment, other predictions of the model are being tested. *T. brucei* cell lines are created in which the expression of HXK and PFK can be manipulated by an exogenous inducer ('conditional knockouts'). These cells will serve to establish if these enzymes are indeed present at high overcapacity, or if the glycolysis model needs additional refinement. These experiments will reveal if the *in vitro* determined kinetic properties of these enzymes apply also to *in vivo* conditions, and/or if additional regulatory mechanisms occurring in the intact cell have to be invoked. Furthermore, the flux control coefficient of triosephosphate isomerase (TIM) has been determined by a combination of genetics and molecular modelling. The modelling shows that the flux decreases when the enzyme activity drops to about 30%. Experimentally it was shown that the growth rate is halved when the enzyme level is decreased to 15%; lower TIM levels are lethal.

It has also been shown that correct compartmentation of enzymes is essential. TIM and PGK are glycosomal enzymes in bloodstream-form trypanosomes. An additional presence, even at low levels, of these enzymes in the cytosol, through expression from a transgenic gene copy, is detrimental for the cell. Computer modelling has provided further insight into the importance for the trypanosome of having intact glycosomes. When, *in silico*, the glycosomal membrane was removed, some glycolytic metabolites may accumulate to very high concentrations as a result of some peculiar kinetic properties of trypanosomal HXK and PFK. These enzymes have evolved for optimal functioning in the specific environment present within the glycosome, but their activity cannot be properly regulated under the conditions pertaining in the cytosol.

4. BIOGENESIS OF GLYCOSOMES

(J. Moyersoen, H. Krazy, V. Hannaert, S. de Walque and P. Michels)

Glycosomes are peroxisome-like organelles into which the matrix proteins are imported by the activity of a variety of proteins called peroxins The translocation of the matrix proteins across the peroxisomal membrane is a complicated cascade process in which different peroxins undergo reversible interactions with each other. Inhibitors interfering with peroxin interactions in trypanosomatids are expected to prevent the synthesis of functional glycosomes and thus kill the parasites. The design of selective inhibitors seems feasible because of the very low level of conservation of peroxins. For the few *T. brucei* peroxins characterised to date identities with human counterparts of only maximally 32 % have been found. We have cloned and characterised two cytosolic peroxins, Pex5 and Pex7, which recognise the PTS1, a signal specified by the three C-terminal amino acids, and the PTS2, a nonapeptide motif close to the N-terminus, respectively. Both *T. brucei* peroxins have been expressed as recombinant proteins in *E. coli*. The functional identity of the 70 kDa TbPex5 has been established *in vitro*; the purified protein recognized *T. brucei* PGK with high affinity. Binding was abolished by cleaving the C-terminal region bearing the PTS1 of PGK. The C-terminal half of TbPex5 comprises seven Tetratrico Peptide Repeat (TPR) motifs. TPR motifs are known to be involved in protein-protein interactions and are found in a considerable number of proteins exerting a variety of different functions in living cells. Colleagues in the USA (Dr. A. Kumar and Prof. W. Hol, University of Washington, Seattle), in collaboration with us, have solved the crystal structure of the first three TPR motifs of *T. brucei* Pex5; the first two motifs appear to adopt a canonical structure, but the third TPR motif has a very unusual, extended conformation never observed before in a TPR protein.

Pex7 belongs to the WD-repeat protein family. These proteins are involved in many cellular functions. WD repeats, units of about 40 residues, are known to mediate protein-protein interactions. TbPex7 is a 40 kDa protein and shares with its homologues from other organisms the presence of six WD repeats.

We have also cloned and sequenced *T. brucei* homologues of Pex6 and Pex14. Pex6 belongs to the family of AAA-proteins (ATPases Associated with a variety of cellular Activities). It is a large (>100 kDa) protein thought to be involved in membrane fusion processes which lead to the formation of mature organelles and possibly also in import of organellar matrix proteins. So far, we have expressed in *E. coli* the separate ATPase domain of TbPex6. It will be used, alongside the full-length protein to be expressed later, for structural and enzymological analysis. Pex14 is associated with the membrane of the organelle. It forms part of the docking complex of Pex5 and Pex7 charged with their cargo proteins. The 39 kDa trypanosome Pex14, and its N-terminal 16 kDa domain, have been expressed as recombinant proteins in *E. coli*. *In vitro* binding studies demonstrated the affinity of the N-terminal domain for the trypanosome Pex5. Crystallisation trials are currently being performed with the various recombinant protein forms of TbPex6 and TbPex14 (J. Choe and Prof. W. Hol).

In addition, two other *T. brucei* proteins possibly involved in glycosome biogenesis have recently been cloned: homologues of Pex10 and Pex12. These peroxins contain a C-terminal zinc-binding RING domain known to be involved in protein-protein interactions.

Our colleagues at the University of Washington (D. Chudzik and Prof. W. Hol), in their collaboration with our group, have also solved the crystal structure of the glycolytic enzyme aldolase, both from *T. brucei* and *L. mexicana*. Trypanosomatid aldolases are characterised by the presence of a PTS2. Interestingly, in the tetrameric aldolase of the parasites, the N-termini from two different subunits, including the PTS2 motifs, form two closely intertwined structures. These "PTS2 dimers", which have very similar conformations in the two aldolase structures, are the first reported conformations of a peroxisomal PTS2. This structural information, together with the recognition of enzyme-specific features on the surface neighbouring the PTS2 dimer, may be exploited for the design of compounds that prevent aldolase from entering glycosomes and thus for the development of selective trypanocidal compounds.

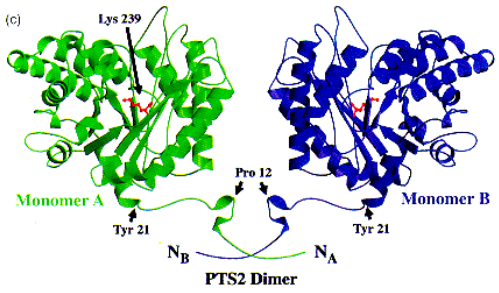

Figure 2. Crystal structure of trypanosomatid aldolase showing the dimer of the PTS2

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IMMUNOGLOBULIN A AND MUCOSAL IMMUNOLOGY

JEAN- PIERRE VAERMAN

Member *: Jean-Pierre VAERMAN* ; **Assistant members** : *Véronique GODDING (part time), Yves SIBILLE (part time), Charles PILETTE, Youssef OUADRHIRI* ; **Graduate Student** : *Didier GIFFROY* ; **Technicians** : *Agnès LANGENDRIES (till October), Françoise DIMANCHE (from September);* **Secretary** : *Anick DELCHAMBRE (part time).*

Human IgA is quantitatively the second Ig in serum, but as shown by our group, the first by its daily synthesis : 70 mg/kg/day vs. 30 for IgG. Over the half of this IgA is destined to exocrine secretions to protect musosae and glands, constituting the secretory immune system, the first line of defense. Another major finding of our group was the very active participation of the hepatocyte in gut immunity in some species such as rat, mouse, hamster and rabbit, but not in others: humans, monkeys, sheep, dogs, and guinea pigs. Hepatocytes express the polymeric Ig receptor (pIgR) also known as secretory component (SC), and actively transport polymeric IgA (pIgA) from plasma into bile against a concentration gradient, keeping plasma pIgA levels low despite continuous supply of large amounts of pIgA from intestinal origin via mesenteric and thoracic duct lymph. The rat hepatocytic transport of pIgA is used as a model by cell biologists who scrutinize the fate and sorting of endosomes, and by immunologists who study secretory IgA (S-IgA) responses after intestinal immunization. For example, we have shown that cholera toxin (CT) given orally to rats induces high S-IgA antibody levels in the bile, which, after derivation into the gut of naive rats, protect them against a CT-challenge. More recently, we showed that binding of pIgA to the pIgR on the basolateral pole of epithelial cells stimulated its transcytosis and apical release, and that J chain in native pIgA was crucial for this receptor-mediated external transport. We also reported an *in vivo* stimulation of pIgR-transcytosis by pIgA-binding in the rat and mouse liver. The src-family protein tyrosine kinase (PTK), $p62^{yes}$, was involved in the control of pIgA transcytosis in mouse bile. We also studied the binding between a pneumococcal surface protein, SpsA, and SC and S-IgA; only human SC, free and in S-IgA, interacted with SpsA, via a small hexapeptide motif.

This year, we report a lack of stimulation of pIgR-transcytosis by pIgA-binding for the human pIgR, strongly suggesting a species difference. We also found a surprizingly strong correlation between reduced expression of epithelial SC (and IgA) in small bronchi, and increasing parameters of airway obstruction and neutrophilic infiltration in patients with chronic obstructive pulmonary disease (COPD), suggesting a possible role of decreased secretory immunity in its pathogenesis. In addition, we examined the *in vitro* effects of interactions of FcαR (CD89) with different molecular sizes of IgA on LPS- and PMA-induced respiratory burst of normal human alveolar macrophages, as well as on their TNF-α release. The involvement of activation of the MAP-kinases ERK1/2 in these effects was also assessed. Finally, we further studied the binding of the pneumococcal SpsA protein to human SIgA, looking for a possible cooperation between SpsA and the IgA1-protease also present on pneumococci, in the cleavage of SIgA1 antibodies.

1. SPECIES DIFFERENCE IN LIGAND-MEDIATED STIMULATION OF pIgR-TRANSCYTOSIS.

(D. Giffroy and J.P. Vaerman, in collaboration with P.J. Courtoy, ICP, CELL)

The intracellular pathway of pIgR is governed by multiple signals leading to constitutive transcytosis. In addition, in transfected polarized MDCK cells, pIgA binding stimulates rabbit pIgR-transcytosis, owing to phospholipase-Cγ1 activation and increase in intracellular calcium. Transcytosis of rat pIgR across hepatocytes into bile is similarly accelerated by i.v. pIgA injection. In contrast, we show here that human pIgR-transcytosis, both in Calu-3 cells which constitutively express pIgR and in human pIgR-transfected MDCK cells, is not promoted by pIgA binding, as monitored by continuous apical release of its secreted ectodomain. However, the incubation of cells expressing human or rabbit pIgR with pIgA induces a comparable inositol-3-phosphate (IP3) production, and pIgR-transcytosis of either species is accelerated by the PKC-activator phorbol myristate acetate (PMA). Without pIgA, mimicking phospholipase-C activation by combining low levels of PMA with the calcium ionophore, ionomycin, or high levels of ionomycin alone, stimulates the rabbit, but not the human, pIgR-transcytosis. These data suggest that the species difference in pIgA-induced pIgR-transcytosis does not stem from the defective production of second messengers, but from a different sensitivity of pIgR to intracellular calcium. Our results also outline the danger of directly extrapolating to humans the wealth of data obtained from the numerous and fashionable attempts at mucosal vaccination performed in laboratory animals.

2. REDUCED EPITHELIAL EXPRESSION OF SECRETORY COMPONENT (SC) IN SMALL AIRWAYS CORRELATES WITH AIRFLOW OBSTRUCTION IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD).

(Y. Sibille and C. Pilette, in collaboration with R. Kiss, Histopathology, ULB, Brussels, and M. Decramer, Pneumology, KUL, Leuven)

Using quantitative immunohistochemistry, we evaluated the expression of SC and Clara cell protein (CC16: marker of bronchial injury) and the neutrophilic infiltration in the airways from eight severe COPD patients undergoing lung transplantation, compared to those from six non-smoking patients transplanted for pulmonary hypertension used as controls (CL), and to lung specimens from five smokers (SM) without chronic bronchitis. The staining for SC was significantly ($p=0.003$) decreased in COPD compared with CL, both in large (with cartilage and/or submucosal glands) and small (unalveolated membranous bronchioles) airways. Moreover, the SC decrease in small airways strongly correlated with impaired functional respiratory parameters, such as forced expiratory volume per sec, forced vital capacity and mean expiratory flow at half vital capacity. In addition, the reduced SC expression in large airways correlated with more neutrophils in submucosal glands ($p=0.03$). CC16 expression in bronchi of COPD was also significantly decreased compared with CL, but without correlation with impaired lung function tests. We plan to investigate if pIgR-KO mice would be more susceptible than their wild type counterparts to a model of COPD induced by SO2-inhalation.

3. SIZE-DEPENDENT EFFECT OF IgA ON LPS- AND PMA-ENHANCED OXIDATIVE BURST AND TNF-α RELEASE BY HUMAN ALVEOLAR MACROPHAGES (HAM). ROLE OF ERK1/2 MITOGEN-ACTIVATED PROTEIN KINASES.

(Y. Ouadrhiri, C. Pilette, and Y. Sibille, in collaboration with R.C. Monteiro, Hôpital Necker, INSERM U25, Paris, France)

HAM express FcαR (CD89) which could link IgA and effector cell branches of the lung secretory immune system. We investigated the effects of FcαR-IgA interactions on LPS- and PMA-induced respiratory burst and TNF-α release by HAM from normal nonsmoking volunteers. Activation of HAM, both with LPS (via a CD14-dependent stimulus) and PMA (receptor-independent stimulus), increased the respiratory burst and TNF-α release through activation of the Extracellular signal-Regulated protein Kinase 1 and 2 (ERK1/2) pathways. Treatment of HAM with PD98059, a selective inhibitor of MAP/ERK kinases (MEK) pathway, inhibited LPS- and PMA-induced oxidative burst and TNF-α release. Polymeric IgA (p-IgA) and secretory IgA (S-IgA) downregulated the LPS-enhanced respiratory burst in HAM through an inhibition of ERK1/2 activity, whereas m-IgA increased both the basal and LPS-induced respiratory burst, both correlating with m-IgA-mediated activation of ERK1/2 kinases. In contrast, all sizes of IgA mediated an increase in the respiratory burst of PMA-

treated HAM. This effect depended on ERK1/2 activation, because IgA upregulated PMA-induced phosphorylation of ERK1/2, and was inhibited by PD98059-treatment of HAM. Moreover, m-IgA, p-IgA and S-IgA increased TNF-α release by HAM through an alternative pathway, distinct from that of ERK1/2 kinases. Our results show a differential regulation of HAM by IgA, through both ERK-dependent and independent pathways. These data may help to understand the still elusive role of IgA in both protective and inflammatory processes in the lung.

4. LACK OF SYNERGY BETWEEN SpsA ON PNEUMOCOCCI AND THEIR IgA1-PROTEASE.

(J.P. Vaerman, in collaboration with M. Delmée, UCL-MBLG, and Sven Hammerschmidt, GBF, Braunschweig, Germany)

SpsA, a choline-binding surface protein present on roughly 75% of all strains of pneumococci, independently of their capsular serotype, interacts specifically with human S-IgA via its SC-moiety, as well as with human free SC, and not with SC nor S-IgA purified from rabbits, rats, mice and guinea pigs. In addition, 93% of pneumococcal strains also express an IgA1-protease, largely as a surface-bound protein, which is thought to represent a virulence factor, because of its ability to cleave secretory IgA1 antibody molecules from human milk into Fab and Fc_ fragments, with ensuing loss of at least some of their antibody activities. We are presently examining if the presence or absence (mutated) of SpsA on pneumococci could influence the kinetics of cleavage of SIgA1 molecules, using both ng amounts of radiolabelled SIgA1 and mg amounts of cold SIgA1 for a given number of viable bacteria. Indeed, it is conceivable that SIgA1 would first be bound to SpsA on pneumococci before being cleaved by the protease; by bringing the substrate closer to the enzyme, this could increase the rate of cleavage. There was however no faster cleavage of a trace of labelled SIgA1 by wild type bacteria than by SpsA mutants. When attempting to reproduce this finding using much larger (mg) amounts of cold SIgA1 for a given number of bacteria, to increase similarity to the *in viv*o situation, there was first no cleavage at all of our polyclonal SIgA1 sample. We showed that this was due to the presence, in our SIgA1, of neutralizing anti-IgA1-protease Abs, because adsorption of our SIgA1 with large numbers of the same bacteria at 4°C and in presence of EDTA, which totally inhibits their IgA1-protease activity, removed the inhibition, now allowing cleavage of the adsorbed SIgA1 sample. It remains to be found if such an adsorbed SIgA1 sample will be entirely cleaved and at the same speed by the same small number of wild type and SpsA-mutated pneumococci.

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VIRAL IMMUNITY AND PATHOGENESIS

JEAN-PAUL COUTELIER

Member *: Jean-Paul COUTELIER;* **Assistant member** *: Françoise CORMONT (part time till August) ;* **Postdoctoral Fellow** *: Karina GOMEZ (from May) ;* **Graduate Students** *: Dominique MARKINE-GORIAYNOFF; Andrej MUSAJI (from May) ;* **Technicians** *: Maria Dolores GONZALEZ, Thérèse BRIET (part time);* **Secretary** *: Anick DELCHAMBRE.*

The possibility for evoluted organisms to survive viral infections depends on the ability of their immune system to eliminate the infectious agent. Therefore, numerous mechanisms, involving different types of immune cells such as cytolytic lymphocytes, T helper and B lymphocytes and macrophages, the molecules that allow those cells to communicate, namely the lymphokines, and the products of those interactions, including antibodies, have been elaborated. On the other hand, viruses have developed strategies to escape the immune system of their hosts, such as large frequencies of mutations or latency, or even to impair this system, which often leads to diseases such as autoimmunity or immunodeficiencies. Our project is to analyze, in murine models, some aspects of these relations between viruses and the immune system.

Of particular interest is the fact that all antibody responses are not equal. Indeed, depending on their isotype, immunoglobulins display various properties. For example, IgG1, one of the major IgG subclass in mice, cannot fix the complement, contrary to IgG2a, another major component of murine immunoglobulins. Such a difference may lead to dramatic variations in the functional effect of antibodies, as their ability to lyse cells they have bound. During the last few years, we found that the isotype of antibody responses was influenced by concomitant viral infections. The effect of the virus resulted in a dramatic increase in the proportion of IgG2a, not only in antiviral antibodies, but also in immunoglobulins with an antigenic target unrelated to viral proteins. In the case of antiviral antibodies, a possible explanation for this phenomenon could be the selection by the infected host of the most appropriate response against the virus. The advantage for the host to select IgG2a in non-antiviral responses is more difficult to understand. In addition, the modification of the isotype of antibodies reacting with self antigens could potentially lead to more deleterious autoimmune reactions. This property of viruses to enhance selectively the production of one immunoglobulin isotype could depend on the preferential activation of a subset of T helper lymphocytes. Indeed, different subpopulations of those cells, called Th1 and Th2, respectively, are distinguished in particular by their capability of producing selectively gamma-interferon or interleukin-4, which can selectively trigger B lymphocytes to produce IgG2a or IgG1, respectively.

In 2000, our analysis of the relationship between viruses and the immune system has focused on a comparison of the efficacy of antiviral antibody IgG subclasses, in a model of neurological disease induced by lactate dehydrogenase-elevating virus (LDV). In addition, we have analysed various factors, including viral infection, that may modify the ability of total IgG to decrease autoantibody-mediated cell destruction.

1. INCREASED PROTECTION BY IgG2a SWITCH MUTANT MONOCLONAL ANTIBODY AGAINST POLIOENCEPHALOMYELITIS INDUCED BY LACTATE DEHYDROGENASE-ELEVATING VIRUS

Neurotropic strains of LDV can induce in immunosuppressed mice of the appropriate genetic background a polioencephalomyelitis leading to paralysis and death of the infected animals. The development of this disease can be prevented by treatment with anti-LDV antibodies. Therefore, LDV-induced polioencephalomyelitis is a useful model for the study of the role of antibody isotype in protection against viral pathogenesis. Thus, IgG1, IgG2a, and IgG2b switch variants were derived from an IgG3 monoclonal antibody directed against the VP3 envelope glycoprotein of LDV. Among the four antibodies, the IgG2a delayed the onset of paralysis and attenuated the severity of the LDV-induced polioencephalomyelitis more than the other subclasses, whereas the IgG3 displayed the strongest neutralizing activity *in vitro*. This suggests that the IgG2a predominance in antiviral antibody responses occuring in the course of infections could be an efficacious way for the host to protect itself.

2. TREATMENT OF AUTOANTIBODY - MEDIATED ERYTHROPHAGO-CYTOSIS BY TOTAL IMMUNO-GLOBULIN: ANALYSIS WITH AN EXPERIMENTAL IN VITRO MODEL OF THE PARAMETERS OF VARIABILITY.

(in collaboration with A. Barclay, Baxter company; T.E. Michaelsen, Department of Vaccinology, National Institute of Public Health, Oslo, Norway; S. Izui, Department of Pathology, Centre Médical Universitaire, Université de Genève, Switzerland)

Several autoimmune diseases, mostly autoantibody-mediated, are attenuated by infusion of total IgG (IVIg). The efficacy varies widely from one patient to another. Using an experimental model of *in vitro* phagocytosis of autoantibody-coated erythrocytes by mouse macrophages, we analyzed the possible causes for such a variability. Our results indicated that the efficacy of the phagocytosis inhibition depends upon different factors such as the isotype and the polymerization of the immunoglobulin used for the treatment as well as the genetic background of the mice and the state of macrophage activation that can be influenced by concomitant viral infection. The development of an *in vitro* assay for the phagocytic activity of macrophages might improve the selection of patients susceptible to benefit from IVIg treatment.

PRIMARY RESEARCH PAPERS

1. Meite M, Léonard S, El Azami El Idrissi M, Izui S, Masson PL, Coutelier J-P. Exacerbation of autoantibody-mediated hemolytic anemia by viral infection. **J Virol** 2000;*74*: 6045-6049.

2. Monteyne P, Coulie PG, Coutelier J-P. Analysis of the Fv1 alleles involved in the susceptibility of mice to lactate dehydrogenase-elevating virus-induced polioencephalo-myelitis. **J Neurovirol** 2000;*6*: 89-93.

3. Gomez KA, Coutelier J-P, Mathieu PA, Lustigand L, Retegui LA. Autoantibodies to cryptic epitopes elicited by infection with lactate dehydrogenase-elevating virus**.** **Scand J Immunol** 2000;*51*: 447-453.

4. Markine-Goriaynoff D, van der Logt JTM, Truyens C, Nguyen TD, Heessen FWA, Bigaignon G, Carlier Y, Coutelier J-P. IFN-γ-independent IgG2a production in mice infected with viruses and parasites. **Intern Immunol** 2000;*12*: 223-230.

THESIS

5. Markine-Goriaynoff D. Doctorat en sciences pharmaceutiques. « Analysis of antibody and T helper lymphocyte modulation by lactate dehydrogenase-elevating virus.

CELLULAR GENETICS UNIT

THIERRY BOON (right)
PIERRE G. COULIE (left)

Members : *Thierry BOON, Pierre G. COULIE;* **Philippe Delori Fellow :** *Vaios KARANIKAS ;* **Postdoctoral fellows :** *Hamid ECHCHAKIR (until September), Panagiota MANTAKA, Francesca SCHIAVETTI, Mitsuhiro TAKENOYAMA, Takeshi HANAGIRI (since September);* **Graduate students :** *Jean-François BAURAIN (until September), Marie-Ange DEMOITIE, Gérald HAMES, Catherine GERMEAU (since September);* **Technicians :** *Thérèse AERTS, Christine MONDOVITS, Catherine MULLER, Bénédicte TOLLET;* **Secretary :** *Suzanne DEPELCHIN.*

Most of our work aimed at understanding by what mechanism tumors were rejected in a few melanoma patients who were vaccinated with a tumor-specific antigenic peptide encoded by a *MAGE* gene. Even though tumor regressions have been observed in a minority of the vaccinated patients several years ago, we were unable to detect a significant increase in the antitumor CTL responses of these patients, even in those who showed a complete and long term regression of several metastases. Considering that one possible explanation for these clinical effects was the involvement of a small number of tumor-specific cytolytic T lymphocytes (CTL), we set up a combination of methods that very much increases the sensitivity of detection of T cells that recognize a precise antigen. Applying this method to some of our responding patients, we eventually detected low level CTL responses.
We also pursued our work on the identification of new tumor antigens, focusing on lung cancer and on antigens presented by HLA class II molecules.

1. DETECTION OF A MONOCLONAL CTL RESPONSE IN A MELANOMA PATIENT VACCINATED WITH AN ANTIGENIC PEPTIDE ENCODED BY GENE MAGE-3.

(V. Karanikas, M.-A. Demoitié, G. Hames, F. Schiavetti and P. Coulie)

Several clinical trials of vaccination with MAGE peptides have been applied to metastatic melanoma patients. In a trial involving three monthly injections with a MAGE-3 peptide presented by HLA-A1, administered in the absence of any adjuvant, we observed that 7 out of the 25 patients who completed the trial showed significant tumor regressions, of which three were complete. Two of these patients, who had regional disease, have remained disease-free for over three years. Several of the regressions started only several months after the first injection and took several additional months to become complete. The rejection of cutaneous nodules occurred in the

absence of strong inflammation. Other trials involving vaccination with MAGE antigenic peptides have confirmed the occurrence of tumor regressions in a minority of patients (our unpublished observations). Tumor regressions have also been observed in advanced melanoma patients immunized with dendritic cells pulsed with MAGE peptides.

Even though the frequency of regressions observed after MAGE vaccination appears to be well above the frequency of the spontaneous regressions that have been observed in metastatic melanoma patients, the possibility of obtaining anti-tumoral responses by vaccinating with peptides alone has met with justifiable skepticism, because it has been generally observed that peptides delivered without adjuvants do not produce CTL responses in mice and can even tolerize in some circumstances. To consolidate the interpretation of the clinical results and to improve the efficacy of the anti-tumoral vaccinations, it is essential to evaluate precisely the anti-MAGE CTL responses elicited by the vaccinations and determine to what extent these CTL responses correlate with the clinical responses. This is difficult because these CTL reponses do not appear to be massive. In one peptide vaccination trial, two patients who showed a complete clinical response were evaluated for their anti-MAGE-3 CTL precursor frequency in the blood. In post-immunization lymphocytes, no evidence was found for a frequency above 3 x 10^{-7} of $CD8^+$, the level observed in a number of non-cancerous individuals. For a trial involving dendritic cells, it was reported that some patients showed post-immunization frequencies of the order of 10^{-4} among $CD8^+$ cells, but there was no clear correlation with clinical responses.

The evaluation of low level anti-MAGE CTL responses is subject to many constraints. Direct measurement of frequency in blood lymphocytes by tetramer analysis is not sufficiently sensitive. Tests carried out with cells that are pulsed with the peptide used for vaccination assay may lead to false positives, due to CTL that are directed against impurities present in the vaccine preparation. Restimulation *in vitro* under limiting dilution can be applied, but it is difficult to assess the exact specificity of the effectors present in the microcultures, unless these are cloned and tested for their lytic activity on MAGE-expressing target cells.

To detect low-level responses, we resorted to antigenic stimulation of blood lymphocyte cultures in limiting dilution conditions, followed by tetramer analysis, cloning of the tetramer-positive cells and T cell receptor (TCR) sequence analysis of the CTL clones that showed strict specificity for the tumor antigen. A monoclonal CTL response against a MAGE-3 antigen was observed in a melanoma patient, who showed a partial rejection of a large metastasis following treatment with a vaccine containing only the tumor-specific antigenic peptide. Tetramer analysis after *in vitro* restimulation indicated that about 1/40,000 post-immunization $CD8^+$ blood lymphocytes were directed against the antigen. The same TCR was present in all the positive microcultures. TCR evaluation carried out directly on blood lymphocytes by PCR amplification led to a similar frequency estimate after immunization, whereas the TCR was not found among 2.5 x 10^6 $CD8^+$ lymphocytes collected before immunization. Our results prove unambiguously that vaccines containing only a tumor-specific antigenic peptide can elicit a CTL response. They suggest that low-level CTL responses can initiate tumor rejection.

25 A TUMOR ANTIGEN PRESENTED BY HLA CLASS II MOLECULES

(R. Chiari, G. Hames, P. G. Coulie, in collaboration with V. Stroobant, Brussels branch of the Ludwig Institute for Cancer Research, and C. Texier and B. Maillère, Département d'ingéniérie et d'Etudes des Protéines, CEA-Saclay, Gif-sur-Yvette, France)

Even though most of the work on the identification of tumor antigens recognized by T lymphocytes on autologous melanoma cells has been conducted with CD8 T cells, which recognize antigenic peptides presented on HLA class I molecules, tumor-specific CD4 lymphocytes may play an important role in antitumor immunity *in vivo*. For example, they were shown to stimulate dendritic cells, which in turn activate cytolytic T cells. This function may be part of the "helper" activity that is often assigned to CD4 T lymphocytes. For an appropriate immunization against cancer, it is therefore possible that CD4 cells that specifically recognize tumor antigens could play a pivotal role for the stimulation of lytic effectors that attack the tumor. Tumor-specific antigenic peptides presented by HLA class II molecules could be encoded by those genes that were already shown to encode peptides presented on tumor cells by HLA class I molecules. The group of P. van der Bruggen identified several peptides that are encoded by *MAGE* genes and that are presented on HLA-DR or -DP molecules to CD4 tumor-specific T cells.

However, it is also possible that the characterization of tumor-specific antigens recognized by CD4 T cells from cancer patients would lead to the identification of new genes that code for tumor-specific antigens.
We obtained a lytic CD4 T cell clone that recognized an antigen presented by HLA-DRB1*1101 on the tumor cells of a melanoma patient who enjoyed an unusually favorable clinical evolution. The antigen appeared to be shared between several melanoma cell lines. To identify the encoding gene we used a new method, based on the cotransfection into human embryonal kidney cell line 293 of a cDNA library from the tumor together with a cDNA clone encoding the class II transactivator, which induces the expression of HLA class II molecules. The product of the gene coding for the antigenic peptide is EphA3, a member of the Eph family of tyrosine kinase receptors, which mediate the repulsion of neural cells by cells carrying the ligand Ephrins on their surface. *EphA3* is expressed at a high level in the retina and fetal brain, at a lower level in several normal tissues, and not at all in hematopoietic cells, the only cells that constitutively express HLA class II molecules. It is overexpressed in several types of tumors, including melanoma, lung carcinoma and sarcoma. On the basis of this pattern of expression, *EphA3* may be a source of tumor-specific antigens recognized on tumor cells that express HLA class II molecules. Anti-EphA3 T cells may have participated in a tumor rejection response in the patient, because the cells of metastases collected several years later than the metastasis used to characterize the antigen had lost expression of *HLA-DR* or *EphA3*, therefore escaping recognition by these lymphocytes.

3. A NEW ANTIGEN RECOGNIZED ON LUNG CANCER CELLS

(J.-F. Baurain, V. Karanikas and P.G. Coulie in collaboration with H. Echchakir and F.-M. Chouaib, Immunologie des Tumeurs Humaines, Institut Gustave Roussy, Paris, France.)

We reported last year on the identification of an antigen presented to autologous CTL by the non-small cell lung carcinoma patient who is enjoying a very long survival after the partial resection of a metastatic tumor. This antigen was encoded by a mutated form of the gene encoding malate dehydrogenase. We pursued our work on the immunogenicity of lung carcinomas, using a CTL clone derived from tumor-infiltrating lymphocytes of another patient with large cell lung carcinoma. We identified the antigen recognized by this CTL clone.
The antigenic peptide is presented by HLA-A2 molecules and is encoded by the *α-actinin-4* gene, which is expressed ubiquitously. In the tumor cells, a point mutation generates an amino acid change that is essential for recognition by the CTL. The mutation was not found in *α-actinin-4* cDNA sequences from about 50 lung carcinoma cell lines, suggesting that it is unique to this patient. Even though the patient did not receive chemotherapy or radiotherapy, he has been without evidence of tumor since the resection of the primary lesion in 1996. Using tetramers of soluble HLA-A2 molecules loaded with the mutated antigenic peptide, anti-α-actinin-4 CTL could be derived from blood samples collected from the patient in 1998 and 2000. It is possible that these CTL, recognizing a truly tumor-specific antigen, play a role in the clinical evolution of this lung cancer patient.

4. EXPRESSION OF GENE MAGE-4 IN REED-STERNBERG CELLS.

(P.G. Coulie, in collaboration with N. van Baren, F. Brasseur, D. Godelaine, Brussels branch of the Ludwig Institute for Cancer Research, H. Chambost, and D. Olive, Institut Paoli-Calmettes, Marseille, France.)

Genes of the *MAGE-A* family are expressed in several types of solid tumors but are silent in normal tissues with the exception of male germline cells, which do not carry HLA molecules.Therefore, peptides encoded by *MAGE-A* genes are strictly tumor-specific antigens that can be recognized by CTL and constitute promising targets for immunotherapy. The expression of 6 genes of the *MAGE-A* family was tested with reverse transcriptase-polymerase chain reaction in lymphoma samples. Among 38 samples of non-Hodgkin lymphoma, 1 anaplastic large cell lymphoma expressed genes *MAGE-A1*, *-A2*, *-A3*, *-A4*, and *-A12*, and 1 lymphoepithelioid T-cell lymphoma expressed gene *MAGE-A4*. Five of 18 samples (28%) from patients with Hodgkin disease expressed gene *MAGE-A4*. In tissue sections, staining by a monoclonal antibody that recognizes the MAGE-A4 protein was observed in 11 of 53 samples (21%) from patients with Hodgkin disease. In the positive samples, the Reed-Sternberg cells were strongly stained whereas the surrounding cells were not. These results indicate that Hodgkin disease may be a target for specific immunotherapy involving MAGE-A4 antigens.

5. A RECOMBINANT HUMAN ANTIBODY THAT SHOWS SPECIFICITY FOR AN HLA/MAGE PEPTIDE COMPLEX.

(P.G. Coulie in collaboration with P. Chames, and H.R. Hoogenboom, Department of Pathology, Maastricht University and University Hospital Maastricht, The Netherlands and A. Ziegler, Institut fur Immungenetik Universitatslinikum Charite, Humboldt-Universitat, Berlin, Germany.)

Antitumor antibodies with the same specificity as CTL that recognize antigenic peptides encoded by *MAGE* genes and presented by MHC class I molecules would be valuable tools to analyze the antigenicity or target tumor cells *in vivo*. To obtain a human antibody directed against a peptide encoded by gene MAGE-1 and presented by HLA-A1 molecules, we selected a large phage Fab antibody repertoire on a recombinant version of the HLA-A1/MAGE-1 complex produced by refolding *in vitro*. One of the selected phage antibodies shows binding to HLA-1 complexed with the MAGE-1 peptide, but does not show binding to HLA-A1 complexed with a peptide encoded by gene *MAGE-3* and differing from the MAGE-1 peptide by only three residues. Phages carrying this recombinant antibody bind to HLA-A1(+) cells only after *in vitro* loading with MAGE-A1 peptide. These results indicate that nonimmunized phage Fab libraries are a source of antibodies with a TCR-like specificity. The human anti-HLA-A1/MAGE-1 antibody described here may prove very useful for monitoring the cell surface expression of these complexes, and possibly, as a targeting reagent for the specific immunotherapy of HLA-A1 patients with a MAGE-1-positive tumor.

PUBLICATIONS

1. Chiari R, Hames G, Stroobant V, Texier C, Maillère B, Boon T and Coulie PG. 2000. Identification of a tumor-specific shared antigen derived from an Eph-receptor and presented to CD4 T cells on HLA class II molecules. **Cancer Res**. *60*:4855-4863.

2. Chambost H, Van Baren N, Brasseur F, Godelaine D, Xerri L, Just Landi S, Theate I, Plumas J, Spagnoli GC, Michel G, Coulie PG and Olive D. 2000. Expression of gene *MAGE-A4* in Reed-Sternberg cells. **Blood** *95*:3530-3533.

3. Chames P, Hufton SE, Coulie PG, Uchanska-Ziegler B and Hoogenboom HR. 2000. Direct selection of a human antibody fragment directed against the tumor T-cell epitope HLA-A1-MAGE-A1 from a nonimmunized phage-Fab library. **Proc. Natl. Acad. Science** *97*:7969-7974.

4. Karanikas V, Colau D, Baurain JF, Chiari R, Thonnard J, Guttierez I, Goffinet C, Van Schaftingen E, Weynants P, Boon T and Coulie PG. 2001. High frequency of CTL directed against a tumor-specific mutated antigen detectable with HLA tetramers in the blood of a lung carcinoma patient with long survival. **Cancer Res**. In press.

5. Baurain JF, Colau D, van Baren N, Landry C, Martelange V, Vikkula M, Boon T and CouliePG. 2000. High frequency of autologous anti-melanoma CTLs directed against an antigen. **J Immunol** *164* :6057-6066.

SEMINARS

January

11 Fabienne VAN de KEERE - Massachusetts Institute of Technology, USA.
Role of the CD4+ cells in experimental autoimmune encephalomyelitis.

20 Stefan CONSTANTINESCU - Whitehead Institute, Cambridge, USA.
The erythropoietin receptor: mechanisms of activation and dowstream signaling.

25 Friedrich FRISCHKNECHT - EMBL Heidelberg, Germany.
Actin-based motility of vaccinia virus.

René REZSOHAZY – GEDE Unit.
Les tribulations d'une protéine Hox : choix de cible et spécificité fonctionnelle.

28 Sabine TÖTEMEYER MIPA Unit.
Effect of *Yersinia enterocolitica* on the inflammatory response.

February

1 Jean-Christophe JONAS – ENDO Unit.
Evaluation du rôle de c-myc myc dans l'hypertrophie et la perte de différenciation des cellules Beta pancréatiques induites par l'hyperglycémie.

4 Mario FELDMAN - Buenos Aires, Argentina.
Flipping of polyprenol-bound sugar : specificity of the translocases and influence of the sugar moieties.

8 Valérie RACE BCHM/GRM Unit.
Corrélations phénotype - génotype dans la déficience en adénylosuccinase.

9 Mohammed ARRAS – TOXI Unit.
IL-9 and experimental pulmonary fibrosis.

10 Marc LEMAITRE & Daniel MARECHAL - Eurogentec BEL S.A., Belgium.
High throughput and quantitative developments in nucleic acids based diagnostic.

11 Pierre FALSON - CEA Saclay, Gif-sur-Yvette, France.
Etude topologique et fonctionnelle de l'ATPase de transport de Ca2+ Sercal.

Howard GOLDFINE - Univ. of Pennsylvania-Medical Center-Philadelphia, USA.
Intracellular signaling in macrophages induced by *Listeria Monocytogenes*.

14 Jean-Paul COUTELIER – MEXP Unit.
Murine polioencephalomyelitis: when genetics, immunosuppression and infection meet to trigger a disease. A litterature overview.

15 Sabine CARDOEN - BCHM/GRM Unit.
Stimulation paradoxale par la 2-chloro-2'-désoxyadénosine de la synthèse d'ADN dans la lignée leucémique humaine EHEB.

18 René REZSOHAZY - GEDE Unit.
Cartographie fonctionnelle de la protéine Hoxa-1.

21 Mojgan RASTEGAR – HORM Unit - Ph.D. thesis in Biomedical Sciences.
The rat hnf6 gene : its structure and control by growth hormone.

22 Mohamed-Rachid BOULASSEL - MBLG Unit.
Maladies auto-immunes de l'oreille interne : caractérisation de certains antigènes cochléaires et induction de la maladie chez le cobaye.

25 Agnès NOEL - Université de Liège, Belgium.
Matrix metalloproteinases expressed by host stromal cells play a critical role in tumor growth.

29 Jean-Paul THISSEN - DIAB Unit.
Mécanismes de résistance à l'hormone de croissance dans le sepsis.

March

1 Christine CLAYTON - ZMBH, Heidelberg, Germany.
Post-transcriptional regulation in trypanosomes: conservation in eucaryotic evolution ?

Stéphane de WALQUE - TROP Unit - Ph.D thesis.
Signals and proteins involved in sorting of glycolytic enzymes to glycosomes of *Trypanosoma brucei.*

Valérie MARTELANGE – LICR.
Identification de SAGE et HAGE, deux nouveaux gènes présentant une expression spécifique dans les tumeurs.

7 Anne DENONCIN - BCHM/GRM Unit.
La protéine régulatrice de la glucokinase, une protéine nucléaire homologue de la glucosamine-6-phosphate-isomérase.

10 Johan DEPREZ – HORM Unit - Ph.D thesis.
Etude de l'activation de la 6-phosphofructo-2-kinase cardiaque par l'insuline.

14 Patrick JACQUEMIN - HORM Unit.
Rôle du facteur de transcription HNF-6 dans la différenciation des cellules endocrines du pancréas.

16 Nathalie CLAUSE - Laboratoire de recherches sur les métastases, Ulg.
Potential involvement of HOX genes in the development of solid tumours.

21 Mustapha AMYERE - CELL Unit.
Dynamique et régulation de la macropinocytose induite par les oncogènes : rôle de la PI3-kinase et de la PI-PLC.

24 Marie-France van den HOVE & Karine CROIZET - CELL Unit.
Régulation de l'endocytose dans les thyrocytes par les Rabs.

28 Gautier DETRY - MIPA Unit..
Inhibition de cascades de signalisation des cellules eucaryotes par les protéines effectrices de *Yersinia.*

April

3 Emmanuel HANON - Imperial College of London, Dept of Immunology, UK.
Fratricide among CD8+ T lymphocytes naturally infected with Human T-cell lymphotropic virus type 1 (HTLV-I).

4 Pascal KIENLEN-CAMPARD - FARL Unit.
Contribution du proteasome à l'expression des gènes du cycle cellulaire dans l'apoptose neuronale.

6 Erik HOOIJBERG - Academic Hospital Free University, Amsterdam, The Netherlands.
Isolation and immortalization of antigen specific human T cells.

David CARLING - Hammersmith Hospital, Imperial College School of Medicine, London, UK.
The AMP-activated protein kinase cascade: a key mechanism for regulating energy metabolism.

19 Jamila LOUAHED – LICR.
Les cellules épithéliales pulmonaires : une nouvelle cible de l'IL-9 dans l'asthme.

21 Rudy BEYAERT - Department of Molecular Biology - RUG/VIB.
Regulatory pathways for NF-kB dependent gene expression.

25 Philippe de DIESBACH - CELL Unit.
L'endocytose des oligonucléotides : purification et caractérisation partielle d'une "protéine réceptrice" sur cellules HepG2.

26 Patricia CORNET - CELL Unit.
Application de la PCR compétitive à l'étude de la régulation de l'expression du messager du TGFb4/EBAF (endometrial bleeding associated factor) dans l'endomètre en culture.

27 Luc BOUWENS - Experimental Pathology Department, VUB.
Transdifferentiation in the pancreas.

Younes ACHOURI – BCHM.
Clonage de l'ADNc de la phosphoglycérate déshydrogénase de foie de rat et étude de la régulation de son expression par les hormones et les nutriments.

Alain JOLIOT - CNRS, Développement et évolution du système nerveux, Ecole Normale Supérieure, Paris, France.
Cellular Biology of Homeoproteins

May

2 Jean-François COLLET - BCHM/GRM Unit.
La phosphosérine phosphatase, modèle d'une nouvelle classe d'enzymes phosphorylés sur un résidu aspartate.

Alain GHYSEN - Laboratoire de Neurogénétique - INSERM 0012 - Université de Montpellier II, Fr.
Du dos de la mouche au flanc du poisson : développement et évolution des systèmes sensoriels.

3 Nathalie DEMOTTE – LICR.
Perte de fonction d'un clone T cytolytique

5 George H. Miller - Microcide Inc, Mountain View, CA, USA.
In vivo essential genes as novel antimicrobial targets.

6 Miikka VIKKULA – BCHM/HMG Unit.
Molecular Genetics of Vascular Anomalies.

9 Christine HALLEUX - ENDO Unit.
Contribution de l'activité sécrétoire du tissu adipeux au syndrome plurimétabolique.

10 Christophe COETSIER - ISTO Unit.
Facteurs environnementaux et génétiques impliqués dans la pathogénèse de la maladie de Crohn.

16 Jean CHRISTOPHE - Laboratoire de Biochimie, Institut de Pharmacie, ULB.
L'obésité : comment atteindre quelques cibles moléculaires ?

17 Luc VAN ROMPAEY - Dept. of Genetics, St. Jude's Chlidrens' Research Hospital, Memphis, TN, USA.
Tel-1/ETV6, a frequent target of leukemic translocations, has tumor suppressor properties.

18 Frédéric LEMAIGRE - HORM Unit.
HNF-6: role in pancreas and liver development.

Isabelle BAR - FUNDP, Namur.
Contrôle de l'expression de la cascade Reelin.

19 Véronique LEFEBVRE - Dept of Molecular Genetics - University of Texas, Houston, USA.
The transcription factors L-Sox5 and Sox6 are required for chondrogenesis

24 Françoise SMETS - IMEX/PEDI Unit.
Defect in interferon-gamma secretion by EBV-specific CD8 T cells associated with EBV-linked lymphoproliferation in immunocompromised children.

25 Bernard PEERS - Laboratoire de biologie moléculaire et de génie génétique, Institut de Chimie, Ulg.
Control of gene expression by the homeodomain factors Pbx in pancreatic cells.

26 Isabelle STAINIER - MIPA Unit.
YscP, a hinge between the "secretion" machinery and the "translocation" apparatus of *Yersinia enterocolitica* ?

30 Sophie LUCAS – LICR.
Identification de nouveaux gènes des familles MAGE et LAGE.

31 Patrice BOQUET - INSERM U.452 - UFR Médecine – Nice, France.
Helicobacter pylori VacA toxin has an activity that is new and surprising for bacterial toxins.

Michel WARNY - MBLG-UCL and Harvard Medical School Gastroenterology division, Boston, USA.
Human immune response to *Clostridium difficile* toxins : role of specific antibodies and mechanisms of monocyte activation.

June

6 Giorgio TRINCHIERI - Schering-Plough.
Antitumor effects of Interleukin-12: role of innate and adaptive immunity.

8 Jean-Paul VINCENT - National Institute for Medical Research, London, UK.
Trafficking of the wingless signal in the embryonic epidermis of Drosophila.

Michel HERMANS - UCL - Unité de Diabétologie et de nutrition.
Comparison of insulin sensitivity and beta-cell function tests in vivo: discriminant ratio and unbiased equivalence.

13 Philippe GHISDAL - UCL - FSIO - Lab. de Pharmacologie expérimentale .
Etude des mécanismes du relâchement endothélium-dépendant et des modifications qui peuvent survenir dans l'hypertension artérielle.

15 Albertina DE SARIO - Institut de Biologie, Montpellier, France.
Régions juxta-centromériques du chromosome 21 : structure génomique et gènes.

16 Thomas MICHIELS – MIPA/VIRO Unit..
Involvement of RNA secondary structure in Picornavirus replication.

22 Gérard GRADWOHL - Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC) - Strasbourg, France.
Function of the bHLH transcription factor neurogenin 3 in entero-endocrine cell differentiation.

23 Sophie BLEVES - MIPA Unit.
Cross-talk between the Yop virulon and the flagellum in *Yersinia enterocolitica*.

26 Simone PARINI - UCL - FSIO - Neural Rehabilitation Engineering.
Mathematical modelling and numerical simulation of artificial nerve fibres activation - Application to optic nerve stimulation.

27 Jacques NEEFJES - The Netherlands Cancer Institute, Amsterdam, The Netherlands.
Movement in living cells to measure alterations in the intracellular peptide pool substrate for MHC class I molecules.

30 Robin MAY - School of Biosciences, University of Birmingham, UK.
The Arp2/3 complex in immunity and invasion.

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LUDWIG INSTITUTE FOR CANCER RESEARCH

BRUSSELS BRANCH

CELLULAR GENETICS

ANNUAL REPORT 2000

STAFF

Boon Thierry, Ph.D., Director, Member

HUMAN TUMOR ANTIGEN GROUP I

van der Bruggen Pierre, Ph.D., Associate Member
Chaux Pascal, Ph.D., Postdoctoral Fellow, to October 2000
Schultz Erwin, M.D., Postdoctoral Fellow, to May 2000
Stroobant Vincent, Ph.D., Postdoctoral Fellow
Van de Corput Lisette, Ph.D., Postdoctoral Fellow, to March 2000
Wildmann Claude, Research Associate
Authom Anne, Technician
Loeuille Sébastien, Technician, from August 2000
Demotte Nathalie, Graduate Student
Lonchay Christophe, M.D., Graduate Student, from October 2000
Panichelli Christophe, M.D., Graduate Student, from October 2000

HUMAN TUMOR ANTIGEN GROUP II

Van den Eynde Benoît, M.D., Ph.D., Associate Member
Bilsborough Jeanine, Ph.D., Postdoctoral Fellow, to June 2000
Chapiro Jacques, M.D., Postdoctoral Fellow
Heidecker Leonora, Ph.D., Postdoctoral Fellow, to September 2000
Ma Wenbin, M.D., Ph.D., Postdoctoral Fellow, from December 2000
Morel Sandra, Ph.D., Postdoctoral Fellow
Pilotte Luc, Research Associate
Piette Fanny, Technician, from September 2000
Maernoudt Anne-Sophie, M.D., Graduate Student, from December 2000
Sonveaux Pierre, Graduate Student, to September 2000

HUMAN TUMOR GENETICS GROUP

De Plaen Etienne, Ph.D., Assistant Member
De Smet Charles, Ph.D., Assistant Member, from October 2000
Lethé Bernard, M.D., Associate Investigator
Chomez Patrick, Ph.D., Postdoctoral Fellow
De Backer Olivier, Ph.D., Postdoctoral Fellow
Auquier Philippe, Technician
Blondiaux Claudine, Technician
Bertrand Mathieu, Graduate Student

Boucquey Marie, Graduate Student
Huijbers Ivo, Graduate Student
Laduron Sandra, Graduate Student
Loriot Axelle, Graduate Student, from October 2000
Martelange Valérie, Graduate Student, to October 2000

MOUSE ANTI-TUMORAL IMMUNIZATION GROUP
Uyttenhove Catherine, Ph.D., Senior Investigator
Warnier Guy, Veterinarian, Associate Investigator
Donckers Dominique, Technician

THERAPEUTIC VACCINATION GROUP
Marchand Marie, M.D., Senior Clinical Investigator
Van Baren Nicolas, M.D., Clinical Investigator
Bejko Dritan, M.D., Clinical Research Associate, from September 2000
Daubresse Pierre, Clinical Research Associate
De La Cuvellerie Ann, Clinical Research Associate, from November 2000
Desaunois Isabelle, Clinical Research Associate, to August 2000
Verfaille Christian, M.D., Clinical Research Associate, from March 2000
Niculescu Liliane, Secretary, from September 2000
Pezechkian Johanna, Secretary, to February 2000
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Brasseur Francis, Ph.D., Associate Investigator
Colau Didier, Ph.D., Associate Investigator
Lurquin Christophe, Ph.D., Associate Investigator
Landry Claire, Ph.D., Assistant Investigator, to December 2000
Huang Lan-Qing, M.D., Postdoctoral Fellow, to January 2000
Lucas Sophie, M.D., Ph.D., Postdoctoral fellow
Zhang Yi, M.D., Postdoctoral Fellow, from January 2000
Ha Thi Vinh, Technician
Letellier Marie-Claire, Technician
Panagiotakopoulos Maria, Technician
Swinarska Magdalena, Technician

Van Malderen Marianne, Technician, to December 2000
Vassilev Vassil, M.D., Graduate Student, from August 2000

CYTOKINE GROUP
Renauld Jean-Christophe, M.D., Ph.D., Associate Member
Van Snick Jacques, M.D., Ph.D., Member, Guest Investigator
Vink Anne, Ph.D., Assistant Investigator
Belladona Maria-Laura, Ph.D., Postdoctoral Fellow, from November 2000
Demoulin Jean-Baptiste, Ph.D., Postdoctoral Fellow, to November 2000
Louahed Jamila, Ph.D., Postdoctoral Fellow, from January 2000
Cormont Françoise, Technician, from September 2000
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Stevens Monique, Technician
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Dumoutier Laure, Graduate Student
Garot Nathalie, Graduate Student, to May 2000
Knoops Laurent, M.D., Graduate Student, from October 2000
Lejeune Diane, Graduate Student
Orabona Ciriana, Graduate Student, to September 2000
Richard Mélisande, Graduate Student

SIGNAL TRANSDUCTION GROUP
Constantinescu Stefan, Ph.D., Assistant Member, from September 2000
Royer Johan, Technician, from September 2000

ANIMAL PRODUCTION GROUP
Warnier Guy, Veterinarian and Animal Group Head
Matthews Jean-Pierre, Technical Assistant
Sirichanto Phone Praseuth, Technical Assistant
Souvannaraj Keota, Technical Assistant

TECHNICAL SUPPORT
Lejeune Joseph, Ph.D., Head of Computer Systems
Mapp Simon, Informatics Assistant, to May 2000
Szikora Jean-Pierre, M.D., Bioinformatics Analyst, to January 2000
Wauters Paul, Laboratory Manager
Negleman Christine, Technical Officer, to June 2000

Tonon André, FACS Operator
Germis Jean-Luc, Technician
Orviz Manuel, Technical Assistant
Van Goetsenhoven Antoinette, Technical Assistant
Hamed Mustafa, Laboratory Helper
Jebari Samira, Laboratory Helper, from January 2000
Mahieu-Gaudy Pascale, Laboratory Helper
Marianini Encarnacion, Laboratory Helper
Reyes Prieto Rafaela, Laboratory Helper
Vandenborre Julien, Office Clerck
Verhelst Cédric, Office Clerck, from September 2000

ADMINISTRATION and SECRETARIAT

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Bien Aline, Accounting and Administrative Assistant
Lemoine Carine, Scientific and Administrative Assistant
Stavaux Pierre, Assistant Accountant
Bodart Marie-Pierre, Scientific Secretary
Krack Nathalie, Secretary
Schoonheydt Geneviève, Secretary

RESEARCH REPORT

Waking up the immune system to eliminate cancer cells efficiently and selectively appears to be an important new approach that may lead to an effective and non-toxic cancer therapy. Past work at the Brussels branch led to the demonstration that most human tumors bear antigens that can be recognized by cytolytic T lymphocytes (CTL) and that are strictly tumor-specific. Some of these antigens are encoded by "cancer-germline" genes, which are expressed only in tumors and germline cells. These antigens are not only strictly tumor specific, they are also shared by many tumors of various histological types. Presently we are pursuing the identification of tumor-specific antigens and we are trying to demonstrate their usefulness in clinical trials involving the therapeutic vaccination of cancer patients.

Two of our research groups are involved in the identification of new human tumor-specific antigens. The group led by Pierre van der Bruggen has focused lately on the task of defining a large number of epitopes encoded by MAGE genes, which constitute a large family of cancer germline genes. A large number of antigenic peptides presented to CTL by either major histocompatibility complex (HLA) class I or class II molecules have been identified on the sequence of the MAGE-1 and the MAGE-3 proteins. This implies that almost every cancer patient whose tumor expresses a MAGE gene will have at least one HLA molecule presenting a MAGE antigenic peptide.

The group led by Benoît Van den Eynde has pursued the observation that some antigenic peptides are processed by the normal proteasome present in tumor cells but not by the immunoproteasome which is present in dendritic cells and in cells treated by interferon-gamma. This finding may have a significant impact on the immunization strategy for some tumor antigens.

The group led by Catherine Uyttenhove pursues the comparison of different modes of immunization with mouse tumor antigens to identify those that produce CTL responses most effectively. Recently, acute toxicity has been observed after immunization with a mouse tumor-specific antigenic peptide.

The therapeutic vaccination group led by Marie Marchand and Nicolas Van Baren is pursuing studies involving immunization with MAGE antigens. A study with the MAGE-3 peptide presented by HLA-A1 has been completed. The observations are: no toxicity and tumor regression in a minority of patients. In other studies involving other MAGE peptides, MAGE-3 protein, and recombinant canarypox ALVAC-MAGE-3, the results appear to be similar to those observed in the first study. New trials involving combination of antigens recognized by $CD8^+$ and $CD4^+$ T lymphocytes will be initiated soon.

Until this year, we were unable to detect any anti-MAGE CTL response in the

patients vaccinated with MAGE-3 antigens, including those patients who showed tumor regressions. We concluded that there was no massive CTL response. A close collaboration between the group of Pierre Coulie (Institute of Cellular Pathology and University of Louvain) and the group of Aline Van Pel has led to the demonstration of a low-level monoclonal anti-MAGE-3 CTL response in a vaccinated patient who showed regression of a large volume of a melanoma metastasis. We are applying to other patients this evaluation approach, which involves *in vitro* stimulation, tetramer analysis, obtention of CTL clones and analysis of their T cell receptor sequences.

The cytokine group led by Jean-Christophe Renauld is pursuing studies on interleukin-9. The importance of IL-9 for mouse resistance to intestinal parasites has recently been demonstrated. To understand better the effect of IL-9, the group has successfully launched a search for mouse genes that are activated in cells treated with IL-9. One of these genes codes for a new cytokine named IL-22. The human equivalent of IL-22 has recently been cloned.

TUMOR ANTIGEN GROUPS

New MAGE antigens recognized by CD8$^+$ and CD4$^+$ T cells.

Efforts to characterize tumor antigens recognized on human tumors by T lymphocytes led to the identification of antigens that are of particular interest for cancer immunotherapy, because of their strict tumoral specificity and because they are shared by many tumors. The first genes identified to code for these antigens were the MAGE genes. Additional cancer-germline genes - i.e. genes that are expressed in tumors and not in normal cells, except in male germline cells - have also been isolated by purely genetic approaches. As a result, a large number of new sequences which have the potential to code for tumor-specific antigens recognized by T lymphocytes are now available. As described below, vaccination of patients with MAGE-3 encoded peptides presented by class I molecules, have produced some tumor regressions. It is therefore important to identify antigenic peptides binding to other HLA class I molecules to permit extension of the approach to a larger group of patients. It is also important to identify MAGE-encoded peptides presented by HLA class II molecules, as the combination of class I and class II peptides may provide superior immunization.

We have designed a general approach for the identification of naturally processed antigenic peptides. CD8$^+$ lymphocytes from non-cancerous blood donors are stimulated in limiting dilution conditions with dendritic cells infected with a canarypox virus (ALVAC) or adenovirus containing the entire MAGE gene. The responder lymphocytes are tested for

specific lytic activity on targets transduced with others vectors carrying the same MAGE sequence. Positive microcultures are cloned to obtain stable CTL clones that are tested on tumor cells. The CTL clones are tested against targets pulsed with a set of overlapping MAGE-encoded peptides to define the antigenic peptides. A similar approach is followed for MAGE antigens presented by class II molecules. Here, $CD4^+$ T cells are stimulated with protein-pulsed dendritic cells.

An important result that was obtained this year is that a MAGE-1and a MAGE-3 peptide known to be presented by HLA-A1 are also presented by HLA-B35. As the MAGE-3.A1 peptide is used extensively in our clinical trials, this will enable us to enlarge the recruitment basis of these trials by adding to the 25 % of patients who express A1, the 19 % who express B35. For MAGE-1, the available antigenic peptides cover 87% of the Caucasian population (*figure 1)*.

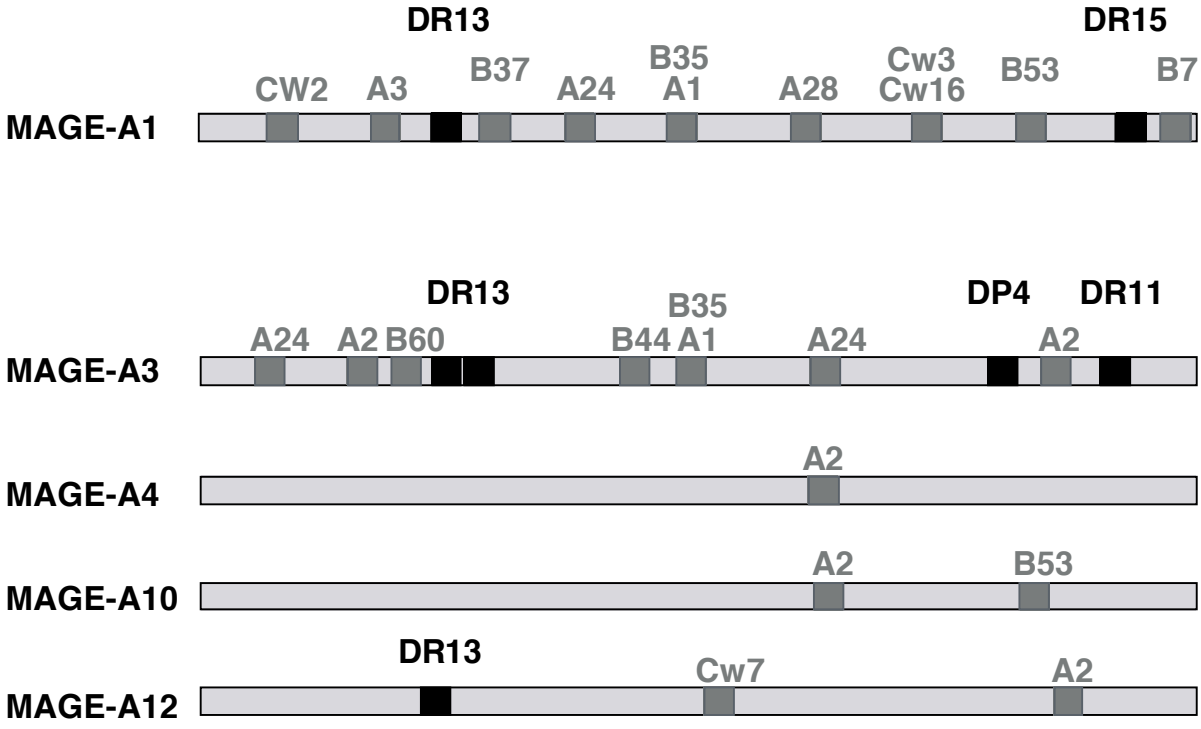

Figure 1

Recently, the exploration of human gene banks has indicated that in addition to the MAGE genes with cancer-germline expression, there exist distantly related MAGE genes that are expressed in all normal tissues. This raised a question about the tumor-specific nature of the antigens encoded by the MAGE genes with cancer-germline expression. However, we found that the ubiquitous MAGE genes are so distant that there is not a single known MAGE antigenic peptide with differences at less than 2 positions from the homologous peptide encoded by an ubiquitous MAGE.

We studied an antigen recognized by a CTL clone, which was raised against a

bladder carcinoma using the classical mixed lymphocyte-tumor cell culture approach. We found that this CTL recognized a new peptide encoded by gene MAGE-A12 and presented by HLA-CW7. This new antigen will be well suited for use in clinical trials because of the very frequent expression of MAGE-12 in various tumor types, and the frequent occurrence of the HLA-CW7 allele.

An antigen encoded by the M-CSF gene

By studying CTL infiltrating a human kidney tumor, we found that they recognize a peptide encoded by an alternative open reading frame (ORF) of the macrophage colony-stimulating factor (M-CSF) gene. Remarkably, this alternative ORF, which is translated in many tumors concurrently with the major ORF, is also translated in some tissues that do not produce M-CSF, such as liver and kidney. Such a dissociation of the translation of two overlapping ORF from the same gene is highly unusual. The antigenic peptide encoded by the alternative ORF is presented by HLA-B*3501 and has a length of 14 residues. Peptide elution indicated that tumor cells naturally present this 14-mer, which is the longest peptide known to be recognized by CTL. Our results suggest that it binds by its two extremities and bulges out of the HLA groove to compensate for its exceptional length.

Loss of specific function of anti-MAGE CTL in vitro.

Human $CD8^+$ CTL clones often lose their specific effector functions, such as cytolytic activity or cytokine production, upon long term *in vitro* culture. We have observed with two CTL clones that this functional defect is induced consistently after repeated stimulation with antigen-bearing EBV-B cells, but not with tumor cells. This defect is correlated with a loss of staining with the relevant fluorescent HLA-peptide tetramer. The CTL still express on their surface T-cell receptor α and β chains and CD3ε, and have an intact lytic machinery as shown by a non-specific lysis assay. Tetramer staining and lytic activity could be recovered after stimulation of tetramer-negative cells with tumor cells in the presence of a supernatant of activated lymphocytes. These results suggest the existence of a new type of functional defect that may involve an impairment in T cell receptor clustering.

Differential processing of some tumor antigens by the standard proteasome and the immunoproteasome.

We previously made the unexpected observation that some class-I-restricted antigens are efficiently processed by the standard proteasome but not by the immunoproteasome, which is induced in most cells upon exposure to interferon-gamma and is expressed in a constitutive manner in dendritic cells. As a result, these antigens are not presented

efficiently by dendritic cells. The first example of such an antigen was an identified peptide recognized on a renal carcinoma by autologous CTL. It turned out to be derived from an ubiquitous self protein that we called RU1. We suggested that the lack of processing of this epitope by the immunoproteasome could explain how the corresponding autoreactive $CD8^+$ T cells escape negative selection in the thymus.

In order to determine whether this observation was unique to the RU1 epitope or was of more general relevance, we undertook a systematic analysis of the processing of a series of tumor epitopes by the two types of proteasome. The analysis of digests obtained after incubation of precursor peptides with either type of purified proteasome indicated that several other antigenic peptides were not processed by the immunoproteasome, including the Melan-A_{26-35}, $gp100_{209-217}$ and $tyrosinase_{369-377}$ epitopes, which are presented by HLA-A2, and the MAGE-$1_{161-169}$ peptide presented by HLA-A1. These results have an important bearing on the choice of an effective strategy of immunization against such antigens in clinical trials, as they imply that better responses should be obtained with immunogens that do not require proteasome digestion in dendritic cells, such as peptides or recombinant vectors containing minigenes.

Not all tumor antigens are poorly processed by the immunoproteasome : several MAGE antigens have been found to be processed equally well by both proteasomes. A new HLA-B60-restricted epitope of MAGE-3 is presented by cells expressing immunoproteasomes, but not by cells carrying standard proteasomes. The standard proteasome appears to destroy the epitope by cleaving within its sequence, whereas the immunoproteasome does not perform this destructive cleavage. Altogether, our observations illustrate the need to analyse carefully the processing of each epitope by the two types of proteasome in order to optimize vaccination with tumor antigens.

HUMAN TUMOR GENETICS GROUP

Function of the MAGE genes

To understand the function of the MAGE genes with cancer-germline expression, we undertook a yeast two-hybrid screen to identify proteins interacting with MAGE-A1. We observed a reproducible binding to nuclear protein SKIP $^{NcoA-62}$, described as a bridging protein between DNA-binding protein CBF1 and a repressor complex containing an histone deacetylase. The intracellular domain of Notch (NotchIC) binds to SKIP $^{NcoA-62}$ and prevents the repressor complex from binding to SKIP. In collaboration with Diane Hayward (Johns

Hopkins School of Medicine, Baltimore), we used Gal4-SKIP in a mammalian two-hybrid assay. We found that MAGE-1 binding to SKIP prevents NotchIC from binding to SKIP, allowing again the repressor complex to bind and exert its function.

MOUSE ANTI-TUMOR IMMUNIZATION GROUP

To enhance anti-P815AB CTL responses induced by injection of the P1A peptide with a human compatible adjuvant based on QS21, a helper sequence called PADRE, for Pan DR epitope, was added to the P1A sequence to form a long colinear peptide. The addition of the PADRE sequence increased the number of responding mice and the level of the CTL response. Similar results were observed with the P198-PADRE peptide. A second sequence was combined to the P1A peptide : 9 amino acids derived from hIL-1β , which exert most of the immunostimulatory activity of the lymphokine but lack its inflammatory, vasoactive and systemic toxic effects. The P1A-IL1 peptide appeared very potent to induce specific CTL responses in mice. As for the PADRE sequence, the position of the IL1 sequence in the colinear peptide is crucial. When added to the C terminal end of the P1A peptide, the PADRE or IL1 sequences improved the immunization. On the contrary, when placed at the NH_2 position, the colinear peptides induced CTL responses equal or lower than those obtained with P1A alone.

Even though the addition of IL-12 to a vaccine composed of the P1A or P198 peptide and a QS21-based adjuvant greatly increased the CTL responses, the addition of IFN-γ had no effect. This observation was surprising since most of the effects of IL-12 have been attributed to the induction of IFN-γ. Consistent with an effect of IL-12 independent of IFN-γ, we observed that addition of exogenous IL-12 greatly increased the CTL response elicited by the P198 peptide and adjuvant in IFN-γ knockout mice.

In the course of our peptide immunization studies in mice, we observed a lethal toxicity after three immunizations of mice with the P1E peptide combined with adjuvant and IL12. This peptide, which binds to H-$2K^d$ to form one of the major antigens recognized by CTL on P815 tumor cells, results from a point mutation in a housekeeping gene named MsrA. The toxic shock observed after peptide immunization appears to result from the production of TNF by P1E-specific CTL, which undergo a massive expansion and represent 30-60% of $CD8^+$ lymphocytes after two immunizations, as measured by staining with H-$2K^d$/P1E tetramer. The toxic response does not result from cross-reactive recognition of the corresponding wild-type MsrA peptide, which also binds to H-$2K^d$. Neither the toxicity nor the massive expansion of specific CTL is observed when mice are immunized with cells transfected with the mutated MsrA gene, although CTL responses of a similar strength are

detected after in vitro stimulation. Tetramer-positive $CD8^+$ clones isolated after two peptide immunizations were found to lyse equally well P815 tumor cells pulsed or not with the P1E peptide, indicating that they recognized the naturally processed antigen and not impurities present in the peptide preparation. However, the same clones produced about ten times more TNF in vitro when they were stimulated with peptide-pulsed P815 cells than with unpulsed P815 cells. The massive expansion of these $CD8^+$ T cells combined with their high production of TNF after peptide stimulation probably explain the toxicity observed after peptide immunization. These results show that precautions must be exerted whenever a new peptide is introduced in human therapy.

THERAPEUTIC VACCINATION GROUP

Therapeutic vaccination with MAGE tumor antigens

We have set up small-scale clinical trials aimed at evaluating the toxicity, the clinical evolution and the immunological response in cancer patients immunized with MAGE vaccines involving either peptides, a recombinant protein or a recombinant viral vector. A total of about 260 patients have been included in these multicentric trials.

Current status of the trials

Fifteen melanoma patients included in the ongoing study LUD 97-004 have received 9 immunizations with the MAGE-3.A1 peptide, injected every 10-11 days. Regressions were observed in two patients, who had a mixed response. As compared with monthly immunizations with the same peptide, which were associated with 7 regressions among 26 evaluable patients, the increase in the vaccination frequency does not seem to improve the clinical benefit. Future patients will receive the same peptide associated with the HLA class II-restricted MAGE-3.DP4 peptide, which may improve the CTL response by the simultaneous activation of specific $CD4^+$ T lymphocytes.

The MAGE-3 protein vaccination study LUD 97-002, which was developed in collaboration with SmithKline Beecham Biologicals and the EORTC, is now completed. Fifty-seven patients with solid tumors, mainly melanoma, were vaccinated with either the MAGE-3 protein alone, or increasing doses of the protein mixed with adjuvant SB AS-2, injected intramuscularly every 3 weeks. Among 36 patients who received at least 4 vaccinations, 5 regressions were noted, without clear relation to either the dose of protein or use of the adjuvant. Thus this vaccine does not seem to induce more regressions than the MAGE-3.A1 peptide, but it does not require that the patient carries a specific HLA type. The clinical efficacy of the MAGE-3 protein injected intradermally (ID) and subcutaneously (SC)

without adjuvant in non-visceral melanoma patients is currently tested in study LUD 99-003. To date, two out of 8 evaluable patients have shown regressions.

In ongoing trial LUD 97-005, 15 patients have received 4 ID and SC immunizations with a recombinant canarypox (ALVAC) virus containing a minigene that encodes the MAGE-1.A1 and MAGE-3.A1 antigens, followed by 3 ID and SC immunizations with the corresponding peptides, at 3-week intervals. Two regressions have been observed. The ALVAC virus is provided by Aventis Pasteur, the sponsor of the trial.

The MAGE-1 and MAGE-3 recombinant proteins will be used to immunize patients with advanced multiple myeloma, who have a stabilized disease after chemotherapy or autologous stem cell graft. Multiple myeloma could be an interesting model for therapeutic immunotherapy, because its evolution is easy to follow by measuring the serum paraprotein concentration, which reflects the tumor burden, and because it offers the opportunity to analyze the bone marrow at several timepoints during vaccination. The study is now open to accrual.

Relevant observations

Immunization with MAGE peptides, the MAGE-3 recombinant protein or the ALVAC recombinant viral vector, is devoid of significant toxicity.

A minority of melanoma patients (about 20 %) show regression of metastatic lesions following immunization. About 10% of the patients show complete or partial clinical responses. Some of them lasted for several years. This frequency is far beyond the reported incidence of spontaneous regressions of melanoma metastases, estimated at 0.2-0.3%, suggesting that these regressions are linked to the vaccinations.

The regression process is often slow to occur and slow to proceed, which suggests that prolonged immunization of patients with a slowly progressing disease might be helpful. Regression of skin metastases is usually not accompanied by local inflammatory reactions. It can be restricted to a subset of the lesions, whereas the others increase in size. Sometimes, new skin nodules can appear during the regression process, then disappear rapidly.

Complete responses can be followed by the appearance of multiple new metastases at new sites in the absence of relapses at the original site. Analysis of some relapsing lesions, or lesions that progressed during mixed responses, has shown persistence of the expression of the MAGE gene that encodes the antigen used in the vaccine. Loss of expression of the MAGE gene is therefore not the main cause of relapse.

Regressions are not restricted to melanoma. A patient with metastatic bladder cancer experienced regression of lymph node metastases after immunization with the MAGE-3 protein.

IMMUNOGENETICS DIAGNOSIS GROUP

Analysis of the T cell responses of vaccinated patients

Even though the frequency of regressions observed after vaccination with MAGE peptide appears to be well above the frequency of the spontaneous regressions that have been observed in metastatic melanoma patients, the possibility of obtaining anti-tumoral responses by vaccinating with peptides alone has met with justifiable skepticism, because it has been generally observed that peptides delivered without adjuvant do not produce CTL responses in mice and can even tolerize in some circumstances. To consolidate the interpretation of the clinical results and to improve the efficacy of the anti-tumoral vaccinations, it is essential to evaluate precisely the anti-MAGE CTL responses elicited by the vaccinations and determine to what extent these CTL responses correlate with the clinical responses. This is difficult because these CTL responses do not appear to be massive. In one peptide vaccination trial, two patients who showed a complete clinical response were evaluated for their anti-MAGE-3. A1 CTL precursor frequency in the blood. In post-immunization lymphocytes, no evidence was found for a frequency above 3×10^{-7} of $CD8^+$cells, the level observed in a number of non-cancerous individuals. For a trial involving dendritic cells, it was reported that some patients showed post-immunization frequencies of the order of 10^{-4} among $CD8^+$ cells, but there was no clear correlation with clinical responses.

To detect low-level responses, we resorted to antigenic stimulation of blood lymphocyte cultures in limiting dilution conditions, followed by tetramer analysis, cloning of the tetramer-positive cells and T cell receptor (TCR) sequence analysis of the CTL clones that showed strict specificity for the tumor antigen. A monoclonal CTL response against a MAGE-3 antigen was observed in a melanoma patient, who showed a partial regression of a large metastasis following treatment with a vaccine containing only the antigenic peptide. Tetramer analysis after *in vitro* restimulation indicated that about 1/40,000 post-immunization $CD8^+$ blood lymphocytes were directed against the antigen. The same TCR was present in all positive microcultures. TCR evaluation carried out directly on blood lymphocytes by PCR amplification led to a similar frequency estimate after immunization, whereas the TCR was not found among 2.5×10^6 $CD8^+$ lymphocytes collected before immunization (*figure 2*).

Recently, we made a similar observation on another vaccinated patient who showed a complete clinical response. These results prove unambiguously that vaccines containing only a tumor-specific antigenic peptide can elicit a CTL response. They also suggest that a remarkably low level of CTL can initiate clinically tumor rejection. However, we do not see a strict correlation between tumor regressions and the occurrence of a CTL response : with

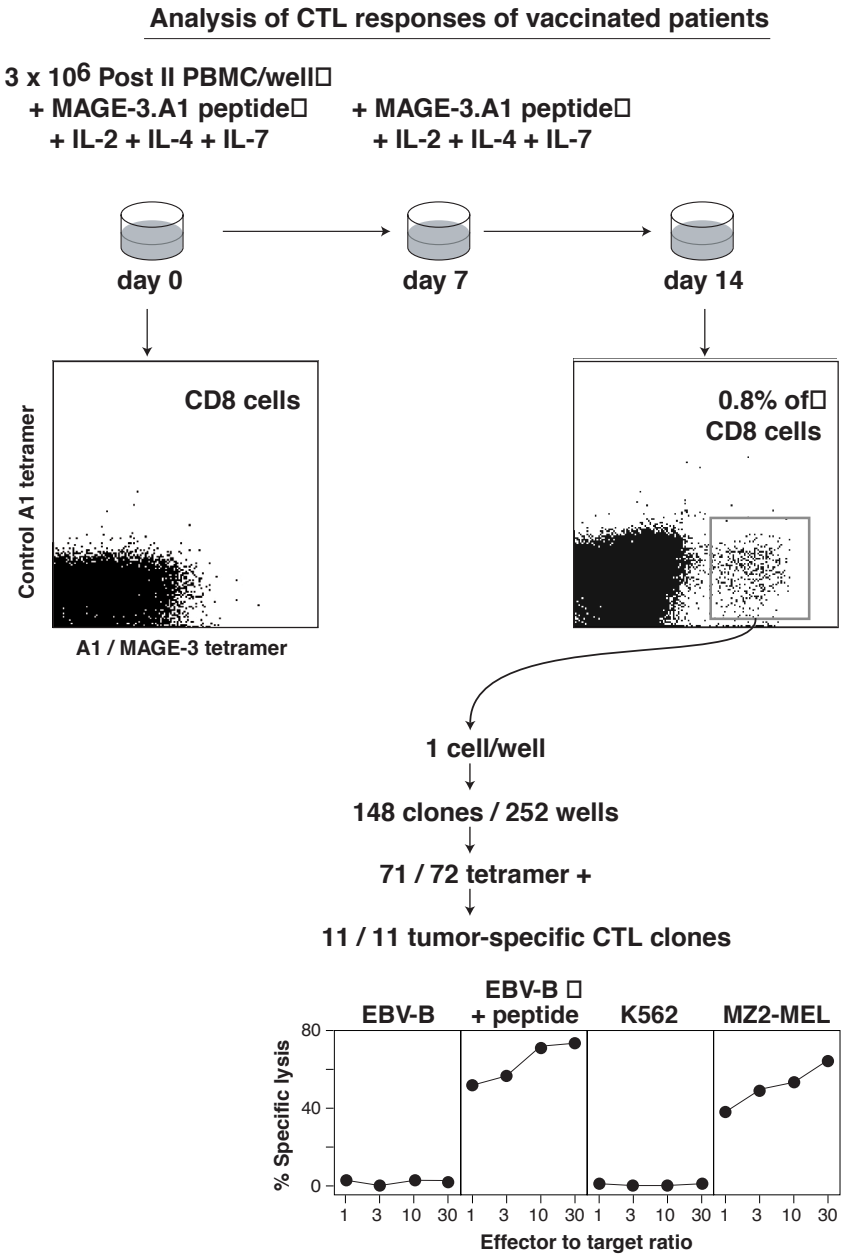

Figure 2

the approach described here, we failed to detect an anti-MAGE CTL response in several patients who showed clinical responses.

CYTOKINES GROUP

Most of the efforts of the cytokine group are focused on the biology of Interleukin-9 (IL-9), a cytokine discovered at the Branch a decade ago. Initial studies focused on its T cell growth factor activity and demonstrated the role of IL-9 in T cell lymphoma development. Recent genetic data in human and mouse models pointed to IL-9 as a candidate gene for

sensitivity to asthma. IL-9 transgenic mice that overexpress this cytokine, show an increase of the processes involved in asthma, including IgE production by B cells, lung mastocytosis, eosinophilia, mucus and chemokine production by lung epithelial cells, as well as bronchial hyperresponsiveness upon exposure to various allergens.

To study further the function of IL-9 *in vivo*, we have immunized a mouse with complexes of IL-9 and ovalbumin. This induced regularly anti-IL-9 autoantibodies. We observed that C57Bl/6 mice, that normally expel the *Trichuris muris* nematode parasite, became susceptible after anti-IL-9 immunization. In addition, neutralization of IL-9 also inhibited parasite-induced blood eosinophilia. These results suggest that IL-9 may play a major role in resistance against intestinal parasites. We also observed that IL-9 confers a protection against septic shock induced by *Pseudomonas aeruginosa* and other bacteria. The protective effect of IL-9 was correlated with a marked decrease in the production of inflammatory mediators TNFα, IL-12, and IFN-γ as well as with the induction of the anti-inflammatory cytokine IL-10.

Upon IL-9 binding, the IL-9R associates with a co-receptor protein called γc. This induces the phosphorylation of the JAK1 and JAK3 tyrosine kinases, which in turn phosphorylate the IL-9R, which then acts as a docking site for STAT-1, 3 and 5. Recent results indicate that transcription factor STAT-5 is responsible for the proliferative effects of IL-9. We observed that T cell lines selected to be independent of IL-9 for their growth have permanently activated STAT-5 present in the nucleus. By contrast, sustained activation of STAT-3 by IL-9 mediates growth arrest of T cell lymphomas.

Among the genes induced by IL-9 in murine T cells, we have identified a new cytokine gene. This gene, initially named IL-TIF and now named IL-22, encodes a protein of 180 amino acids that shows homology to IL-10. Moreover, IL-22 shares the second chain of its receptor with IL-10. We have now cloned the human IL-22 equivalent, which is located on chromosome 12q15, near the IFN-γ gene, in a region that has been associated to allergic asthma and inflammatory bowel disease. Despite its similarities with IL-10, IL-22 does not exert the same biological activities. It is induced by LPS injection *in vivo*, and promotes the production of acute phase proteins by liver cells. Contrary to IL-10, it does not inhibit macrophage activation during inflammation.

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