Research at

the Christian de Duve Institute of Cellular Pathology
(ICP)

and at

the Brussels Branch of
the Ludwig Institute for Cancer Research
(LICR)

2003

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Christian de Duve Institute of Cellular Pathology (ICP)

ICP: an international biomedical research Institute

When Christian de Duve founded the Institute of cellular Pathology (ICP), in 1974 he was acutely aware of the contrast between the enormous progress in biological sciences that had occurred in the 20 preceding years and the modesty of the medical advances that had followed. He therefore created a research institution based on the principle that basic research in biology would be pursued by the investigators with complete freedom, but that special attention would be paid to the exploitation of basic advances for medical progress. It was therefore highly appropriate for the Institute to be located on the campus of the Faculty of Medicine of the University of Louvain (UCL). This campus is located in Brussels. The University hospital (Cliniques St Luc) is located within walking distance of ICP.

Thierry Boon

Emile Van Schaftingen

The main commitment of the members of ICP is research. Discovery is the endpoint of their effort and the only element taken into account for their evaluation. But the Institute functions in symbiosis with the Faculty of Medicine and many of its senior members hold Faculty position and have teaching appointments. The influx of doctoral students and postdoctoral fellows from the University is also a key element in the success of the Institute.

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In 1978 the Ludwig Institute for Cancer Research decided to base its Belgian branch within the walls of ICP. A happy collaboration between the two Institutions has been pursued since that time. Even though the two Institutes are completely independent, the collaborations between the scientists of ICP and the Ludwig Institute is extremely close and the sharing of resources is considerable

ICP is managed by a directorate of three scientists, presently composed of Thierry Boon, Fred Opperdoes and Émile Van Schaftingen. The directorate is appointed by the Board of directors, which comprises the Rector of the University of Louvain; the Pro-rector, the General Administrator of the University and the Dean of the Faculty of Medicine. Also present in the Board of directors are eminent members, of the business community.

About 150 researchers work in ICP and in the Ludwig Institute, assisted by a technical and administrative staff of about 100 members. Despite this relatively small size ICP has the ambition of pursuing research projects of high quality under conditions that allow original, long-term projects to be pursued. The Institute has a limited endowment that is a source of key financing for priority issues, such as the creation of new laboratories for promising young researchers. We expect that the quality of our researchers supported sound organisational approaches will enable ICP to stand at the forefront of the European Research Institution.

Fred Opperdoes

The ICP is an autonomous non-profit international association (Association internationale sans but lucratif, AISBL), endowed with its own legal personality.

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For many years, the interest of our group has been focused on the regulation of carbohydrate metabolism, two important contributions being the discovery of fructose 2,6-bisphosphate (in 1980, in collaboration with L Hue, HORM unit, and HG Hers, former head of this group; 1) and that of the regulatory protein of glucokinase (in 1989; 2). We have also kept an interest in inborn errors of metabolism and our lab has contributed the identification of several "new" enzymatic deficiencies, including that of phosphomannomutase (in collaboration with Prof. J. Jaeken, Leuven; 3). As a result of this, part of our work has been devoted to the biochemical characterization of some of the enzymes involved in these deficiencies. More recently, the study of the mechanism of formation of the intriguing phosphate ester, fructose 3-phosphate, has led us to identify fructosamine 3-kinase(4). This has brought us into very different fields, those of protein repair and diabetic complications.

Molecular studies on glucokinase and its regulatory protein

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

Glucokinase is the enzyme that phosphorylates glucose in the liver and in the ß-cells of pancreatic islets. Contrary to other hexokinases, it displays a low affinity for glucose and is not inhibited by physiological concentrations of glucose 6-phosphate. In 1988 our group discovered in rat liver a regulatory protein (GKRP) that inhibits glucokinase competitively with respect to glucose. The effect of GKRP is reinforced by fructose-6-P (F6P) and antagonized by fructose-1-P (F1P), which act therefore as glucokinase inhibitor and activator, respectively (Fig.1). These effectors act by binding to GKRP and by modulating its affinity for glucokinase, presumably by promoting distinct conformational changes of GKRP (represented by squares and circles in Fig. 1). The effect of fructose-1-P accounts for the fact that fructose stimulates glucose phosphorylation in rat liver. Fructose can therefore serve as a nutritional signal telling the liver that simple sugars are taken up by the intestine.

Recent efforts on this subject have been devoted to the identification of GKRP residues involved in the binding of fructose 6-phosphate and fructose 1-phosphate (5). GKRP is homologous to bacterial proteins of unknown

function, as well as, more distantly, to glucosamine-6-P synthase (GLMS). This enzyme, which uses fructose 6-phosphate and glutamine to produce glucosamine 6-phosphate and glutamate, contains a “glutaminase domain” as well as an “isomerase” domain to which GKRP is homologous. This homology enabled us to identify GKRP residues that could potentially interact with fructose-6-P. Mutations of several of these residues led to proteins that had a reduced effect on glucokinase and that had much less affinity not only for fructose 6-phosphate but also for fructose 1-phosphate. These results indicate that GKRP has one single binding site for fructose-6-P and fructose-1-P. This binding site is predicted to be at the interface of two “SIS subdomains” similar to those found in GLMS.

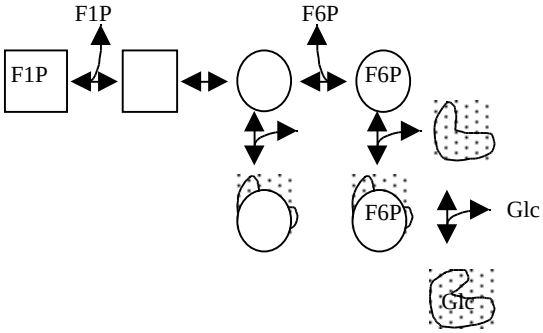

Fig.1.

Glucose-6-phosphatase

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

One of the important functions of the liver and of the kidneys is to produce glucose. This ability critically depends on the presence of glucose-6-phosphatase, an enzyme of the endoplasmic reticulum that hydrolyses glucose-6-P to glucose and inorganic phosphate. According to the substrate-transport model, glucose-6-phosphatase is a rather non-specific hydrolase, whose catalytic site is oriented towards the lumen of the endoplasmic reticulum. Glucose-6-P is transferred from the cytosol to the lumen of the endoplasmic reticulum by a specific transporter (Fig. 2), which confers to glucose-6-phosphatase its specificity. This model accounts for several observations, including the fact that chlorogenic acid and some of its derivatives (like S3483, used in the studies described below) inhibit the hydrolysis of glucose-6-P in intact but not in detergent-treated microsomes. These compounds are thought to be inhibitors of the glucose-6-P translocase. We reported in 1997 the cloning of a cDNA encoding a putative translocase (6) and showed that it is mutated in glycogen storage disease type Ib (7), in which there appears to be a defect in the microsomal transport of glucose-6-P.

Despite these and other findings, the substrate-transport model is not unanimously accepted. Recent work has been therefore devoted to the demonstration of the occurrence of glucose-6-P transport into liver microsomes. In one study, we have taken advantage of the fact that glucose-6-phosphatase catalyses the synthesis of glucose-6-P when microsomes are incubated in the presence of glucose and a phosphate donor such as carbamoyl-phosphate. Under these conditions, we found that S3483 caused the intravesicular accumulation of glucose-6-P and inhibited the appearance of extravesicular glucose-6-P (8).

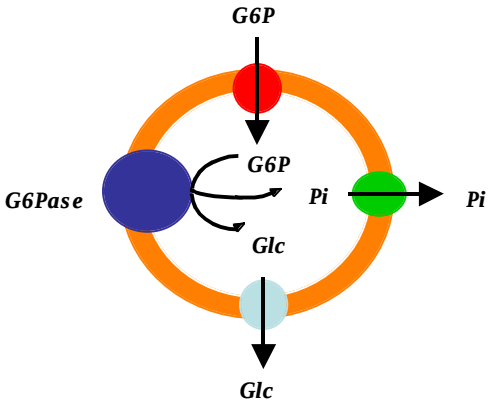

Fig.2.

This result indicates that the catalytic site of glucose-6-phosphatase is oriented towards the lumen of microsomes. In another study, we showed that S3483 inhibits the conversion of glucose-6-P to 6-phosphogluconate that occurs inside microsomes in the presence of electron acceptors such as NADP or metyrapone. This reaction is catalysed by hexose-6-phosphate dehydrogenase, a form of glucose-6-P dehydrogenase that is present in the endoplasmic reticulum. Inhibition of the oxidation of glucose-6-P by S3483 indicates that the glucose-6-P transporter furnishes substrate not only to glucose-6-phosphatase, but also to hexose-6-phosphate dehydrogenase. These findings are further proof for the substrate-transport model.

Fructosamine metabolism

Gh. Delpierre, F. Collard, J. Fortpied, E. Wiame, E. Van Schaftingen

Chronic elevation of the blood glucose concentration in diabetes appears to be responsible for the long-term complications of this disease. The link between the elevated concentration of glucose and the development of these complications is not yet clear. One of the theories on this link emphasises the role of fructosamines.

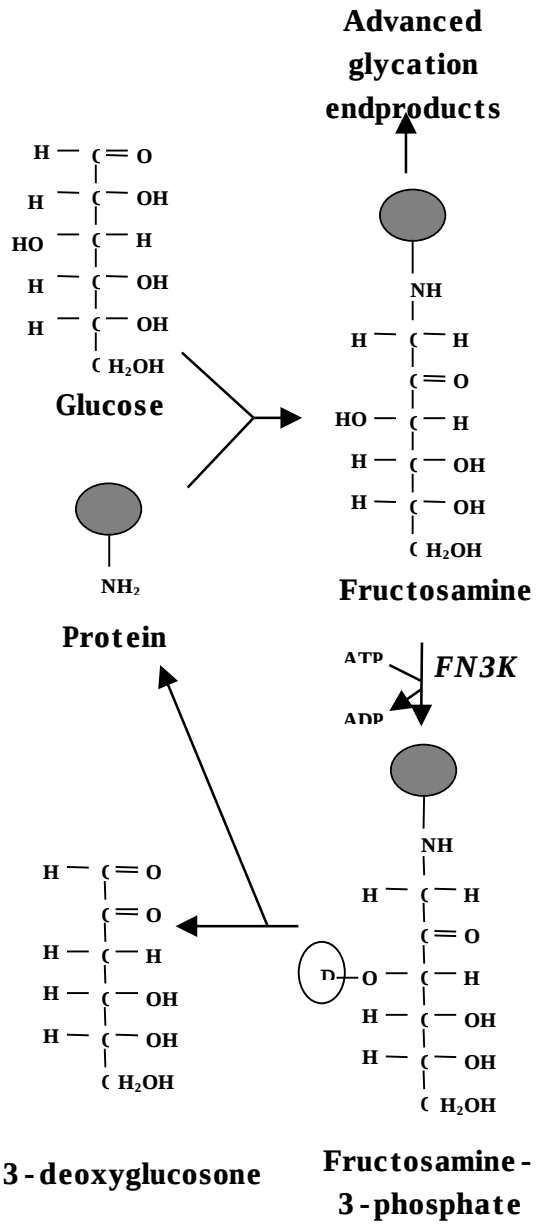

Fig.3.

We have now found that incubation of human erythrocytes with 200 mM glucose not only caused the progressive formation of glycated haemoglobin, but also increased the level of an anionic form of haemoglobin containing alkali-labile phosphate, to about 5 % of total haemoglobin. Deoxymorpholinofructose (DMF), a competitive inhibitor of fructosamine-3-kinase, increased twofold the rate of accumulation of glycated haemoglobin, but markedly decreased the amount of haemoglobin containing alkali-labile phosphate. We could therefore conclude that the latter corresponds to haemoglobin with a fructosamine-3-P group (FN3P-Hb). Returning erythrocytes incubated with 200 mM glucose and DMF to a low-glucose medium devoid of DMF caused a decrease in the amount of glycated haemoglobin, a transient increase in FN3P-Hb and a net decrease in the sum (glycated haemoglobin + FN3P-Hb). These effects were prevented by DMF, indicating that fructosamine-3-kinase is involved in the removal of fructosamine residues. The second step of this “deglycation” process is most likely a spontaneous decomposition of the fructosamine-3-P residues to a free amine, 3-deoxyglucosone and Pi. This is supported by the finding that 2-keto-3-deoxygluconate, the product of 3-deoxyglucosone oxidation, is formed in erythrocytes incubated for two days with 200 mM glucose in sufficient amount to account for the removal of fructosamine residues from proteins (9).

While overexpressing mammalian fructosamine 3-kinase *in Escherichia coli*, we noted that control bacterial extracts contained low activities of an enzyme capable of phosphorylating fructoselysine. This stimulated us to study the metabolism of fructoselysine in *E. coli*. We found that these bacteria indeed grow on fructoselysine as an energetic substrate at a rate of about 1/3 of that observed with glucose. Extracts of cells grown on fructoselysine catalysed the ATP-dependent phosphorylation of fructoselysine to a product that was converted to hexose phosphates and other phosphorylated intermediates. Database searches allowed us to identify an operon containing a putative kinase (YhfQ) belonging to the PfkB/ribokinase family, a putative deglycase (YhfN), homologous to the isomerase domain of glucosamine-6-P synthase and a putative cationic amino acid transporter (YhfM). The proteins encoded by YhfQ and YhfN were overexpressed in *E. coli*, purified and shown to catalyse the ATP-dependent phosphorylation of fructoselysine to fructoselysine-6-P (YhfQ), and the reversible conversion of fructoselysine-6-P and water to lysine and glucose-6-P (YhfN). The kinase and

the deglycase were both induced when *E. coli* was grown on fructoselysine, and reached then activities sufficient to account for the rate of fructoselysine utilization. The two-step pathway that we have identified accounts therefore for fructoselysine utilisation by *E. coli*. Free fructoselysine is most probably produced through digestion of glycated protein. It is apparently not absorbed by the gut, but degraded by the microbial flora in the intestine. Our results indicate that *E. coli* participates in this metabolism.

Reaction mechanism of phosphoglycerate mutase

J.F. Collet, E. Van Schaftingen in collaboration with V. Stroobant, Ludwig Institute

The conversion of 3-phosphoglycerate to 2-phosphoglycerate is catalysed by two types of phosphoglycerate mutases (PGM), which differ in their mechanism, amino acid sequence and three-dimensional structure. The first type, present in vertebrates, certain invertebrates, yeast, fungi and some bacteria, uses 2,3-bisphoglycerate as a cofactor. The catalysed reaction is inter-molecular and follows a ping-pong mechanism in which a histidine residue is phosphorylated. By contrast, the cofactor-independent PGM (PGMi) does not require 2,3-bisphosphoglycerate. It is found in plants, in some microorganisms including bacteria and *Trypanosoma brucei*. It is a monomeric enzyme of about 60 kDa that catalyses an intramolecular reaction and requires a divalent metal cation for activity. Several data plead for the formation of a phosphoenzyme intermediate, which has, however, not been isolated until now.

We have now reported (10) that recombinant, cofactor-independent phosphoglycerate mutase from *T. brucei*, is inactivated by incubation with EDTA and reactivated by Co^{2+} much more than by Mn^{2+} or Fe^{2+}. It displays a minor (maximally 0.01 % of the mutase activity) phosphoglycerate phosphatase activity, which is stimulated by Mn^{2+} more than by Co^{2+} and displays a K_M of 5 µM in the presence of Mn^{2+}. Upon incubation with [^{32}P]-phosphoglycerate, radioactivity was incorporated into the enzyme; this reaction was stimulated by Mn^{2+} and Fe^{2+} much more than by Co^{2+}. The phosphorylated residue was identified by tandem mass spectrometry as Ser74, a residue in homologous position as the phosphorylated serine in alkaline phosphatase. The formation of the radiolabelled phosphoenzyme and its disappearance upon addition of cold substrate were slow events that took several minutes to be complete, contrasting with the kcat of the mutase reaction (28 s^{-1}). The K_M for the formation of the phosphoenzyme (5 µM) was about 20-fold lower than the K_M for 3-phosphoglycerate in the mutase reaction, but similar to the value observed with the phosphatase activity. These results indicate that the observed phosphoenzyme is an intermediate in the minor phosphatase activity, but not in the phosphomutase reaction.

Inborn errors of metabolism

T. de Barsy, E. Van Schaftingen

In 2001, samples from about 100 patients were analysed, allowing the diagnosis of carnitine deficiency (3 cases), of aldolase B deficiency (2 cases), of various forms of glycogen storage disease (6 cases) and of phosphomannomutase deficiency (20 cases).

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PURINE METABOLISM

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Purine metabolism is essential to the body: it provides components of the nucleic acids, DNA and RNA, and the energy currency of the cell, ATP. Purine catabolism leads to the formation of a poorly soluble compound, uric acid, which can precipitate when elevated, and thereby causes gout. Our major present interests are the genetic defects of purine metabolism, and the mechanisms of action of select synthetic purine nucleoside analogues which possess therapeutic, mainly anticancer and antiviral properties.

In previous work we demonstrated 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. Interruption of this recycling plays a crucial role in the elevation of adenosine, a major regulatory compound, under anoxic conditions. We have also shown 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. Furthermore, we have discovered that phosphorylation of aminoimidazolecarboxamide (AICA) riboside into the corresponding nucleotide, AICA-ribotide (AICAR, also termed ZMP), an intermediate of the 'de novo' pathway, results in the inhibition of key glycolytic and gluconeogenic enzymes in the liver owing to its resemblance with AMP. We have also found that ZMP 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 (1).

Adenylosuccinate lyase deficiency

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

Collaboration with the Department of Paediatrics of the University Hospital Gasthuisberg in Leuven has led us to the discovery, in 1984, of adenylosuccinate lyase (adenylosuccinase, ADSL) deficiency, the first enzyme deficiency described on the 'de novo' pathway of purine synthesis in man. This disorder causes accumulation in cerebrospinal fluid and urine of two normally undetectable compounds, succinylaminoimidazolecarboxamide riboside (SAICA-riboside) and succinyladenosine (S-Ado). These are the dephosphorylated derivatives of the two substrates of ADSL, SAICA-ribotide (SAICAR) and adenylosuccinate (S-AMP), respectively (see Figure). Affected children display variable, but mostly profound psychomotor delay, often epilepsy and/or autistic features, occasionally growth retardation and muscular wasting (2). We study the mutations that lead to ADSL deficiency (3-5), and the pathophysiologic mechanisms of the disorder.

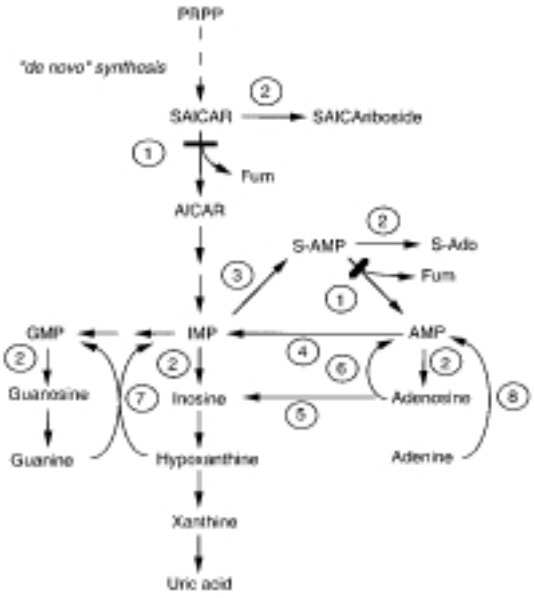

Fig. 1. Pathways of purine metabolism. The *ten-step synthetic route*, often termed 'de novo' pathway, leads from phosphoribosyl pyrophosphate (PRPP) to IMP. From IMP, the nucleoside monophosphates, AMP and GMP, and the corresponding di- and triphosphates (not shown) are formed. The *catabolic pathway* starts from the nucleoside monophosphates and, in man, produces uric acid, a poorly soluble 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. AICAR, aminoimidazolecarboxamide ribotide; Fum, fumarate, S-Ado, succinyladenosine; SAICAR, succinylaminoimidazolecarboxamide ribotide; S-AMP, adenylosuccinate. (1) adenylosuccinate lyase; (2) cytosolic 5'-nucleotidase; (3) adenylosuccinate synthetase; (4) AMP deaminase; (5) adenosine deaminase; (6) adenosine kinase; (7) hypoxanthine-guanine phosphoribosyltransferase; (8) adenine phosphoribosyltransferase. Bars indicate the defect in adenylosuccinate lyase deficiency.

Mutation analysis

ADSL deficiency has been diagnosed in more than 60 patients worldwide. In accordance with the variability of the clinical picture, 38 different mutations in the ADSL gene have been identified to date in 40 unrelated families (see Adenylosuccinate Lyase Mutations Database at http://www.icp.ucl.ac.be/adsldb/). The majority are missense mutations. 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 5 families.

A - 49 T>C change in the 5' untranslated region (5'UTR) of the ADSL gene, found in 2000 in an ADSL-deficient patient in whom one of the ADSL genes displayed a normal coding sequence, was diagnosed in two additional unrelated patients. Studies of the influence of this mutation on gene expression (5) confirmed that it provokes 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 investigation has shown that the mutation affects the binding of NRF-2, a known activator of transcription. The functional importance of the mutation was corroborated by gel-shift experiments that showed that RCO4-NRF-2 oligonucleotides competed very well the WT-ADSL oligonucleotide but not the ADSL oligonucleotide with the - 49 T>C mutation. The observation that, in binding assays with nuclear proteins, the oligonucleotide probe with the – 49 T>C mutation revealed a complex of higher molecular weight, suggests that the mutation might also allow the binding of an unknown repressing factor. Our findings indicate that a mutation of a regulatory region of the ADSL gene might be an unusually frequent cause of ADSL deficiency, and suggest a role for NRF-2 in the gene regulation of the purine biosynthetic pathway.

Pathogenetic studies

The symptoms of ADSL deficiency could a priori be due to a distal deficiency of purine, particularly adenine nucleotides, and/or to a toxic effect of proximally accumulating SAICAR and S-AMP, and/or of their

dephosphorylated derivatives, SAICA-riboside and S-Ado, respectively.

The observation that the levels of SAICA-riboside are comparable in severely and mildly retarded patients, whereas those of S-Ado are markedly higher in the latter, has led to the hypothesis that SAICA-riboside is the neurotoxic compound, and that S-Ado could counteract its noxious effects. However, we could not demonstrate a cytoxic effect of the succinylpurines on cultured rat neurones. A recent search for interactions of the succinylpurines with 30 membrane receptors by binding analysis has also given negative results.

These findings have led us to consider the possibility that the intracellular accumulation of the substrates of the enzyme might be toxic. To test this hypothesis, construction of ADSL-deficient models was initiated. Several approaches are under way to obtain ADSL-deficient cells: creation of dominant negative mutants by overexpression of mutated ADSL in mammalian cells, expression of antisense RNAs to repress endogenous ADSL, and inhibition of ADSL by compounds such as adenylophosphonopropionate. We are also in the process of creating an ADSL-deficient neuronal cell line by knock-in through insertion of the most frequent mutation, R426H, in the murine gene. Mouse N1E-115 neuroblastoma cells have been efficiently transfected with the modified ADSL gene using lipofection, and we are screening the neomycin-resistant recombinant clones for homologous recombination.

Anti-leukaemic 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

In 1997, a collaborative study of the antileukaemic nucleoside, 2-chloro-2'-deoxy-adenosine (CdA), was started with the Department of Haematology of the University Hospital Saint-Luc. This adenosine deaminase-resistant deoxyadenosine analogue displays remarkable therapeutic properties in indolent lymphoid malignancies including hairy cell leukaemia and B-cell chronic lymphocytic leukaemia (B-CLL). 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 compounds (6).

To exert is antileukaemic 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. 2-Chloroadenine, the major catabolite of CdA, was found to be actively phosphorylated, but poorly cytotoxic (7).

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. This cell line was found resistant to CdA, and the mechanisms responsible for this resistance are now analyzed. We have also initiated a study of the regulation of dCK, a key enzyme in the activation of CdA and of several other nucleoside analogues used in anticancer or antiviral therapy.

Resistance to CdA of EHEB cells

The EHEB cell line was found to be less sensitive (10- to 1000-fold) to the nucleoside analogue CdA than other human lymphoblastic cell lines. Moreover, DNA synthesis, measured by thymidine incorporation into DNA, was unexpectedly increased in EHEB cells, up to 2-fold, after a 24 h-incubation with 10 µM CdA (8). Analysis by flow cytometry, using double labelling with propidium iodide and bromodeoxyuridine, has shown that CdA, in EHEB cells, provokes an increase in the proportion of cells in S phase, synthesizing actively DNA. These results contrast with those reported in other leukemic cell lines sensitive to CdA, like CCRF-CEM cells, in which CdA inhibits DNA synthesis and provokes an accumulation of most cells in either early S phase or at the G1-S border. Kinetics and synchronisation experiments have shown that CdA stimulates the progression of EHEB cells from G1 to S phase, rather than blocking them in S phase. This led us to study the effect of CdA on proteins regulating the G1/S checkpoint of the cell cycle, and firstly on the phosphorylation of the retinoblastoma (Rb) protein, which is increased during the

G1/S transition. We have observed that CdA enhances the phosphorylation of Rb in EHEB cells, whereas it decreases it in CCRF-CEM cells. The p53 status of this cell line was determined and found unmutated. Additional preliminary experiments have shown that CdA decreases p21 expression in EHEB cells. In conclusion, we show a new mode of cellular response to CdA, implying modification of the cell cycle regulation leading to enhanced DNA synthesis. We propose that this peculiar effect might be implied in some types of yet unexplained resistance of leukaemic cells to CdA.

Regulation of dCK activity

Since dCK activates numerous nucleoside analogues used in anticancer and antiviral therapy, knowledge of its regulation can be expected to allow optimization of the activation of these analogues. Recently, it has been shown by others and by us that dCK activity can be increased by various genotoxic agents, including CdA, aphidicolin, etoposide, and UV-C irradiation. This activation is not explained by an allosteric effect or by an increase of the quantity of enzyme. A post-translational activation of dCK by intracellular signalling pathways was suggested. To unravel the mechanism of the activation of dCK by CdA, we first investigated the effect of a variety of activators and inhibitors of protein kinases on the basal activity of dCK and on its activation by CdA. We discovered that dCK can be activated by several inhibitors of protein kinases, including genistein, an unspecific inhibitor of protein tyrosine kinases, AG-490, an inhibitor of the protein tyrosine kinase JAK-2, PD-98059, U0126, SB 203580 and dicoumarol, which are inhibitors of various MAP kinases. We also observed that these inhibitors potentiated the activating effect of CdA. In contrast, sorbitol which is known to activate the MAP kinase pathway was found to decrease dCK activity and to counteract its activation by CdA. On the other hand, we have shown that dCK activity can be markedly increased in intact EHEB cells by incubation with okadaic acid, an inhibitor of protein phosphatase PP2A. Taken together, these results do not allow to identify the pathway by which dCK is activated, but clearly indicate that its activity can be regulated by protein kinase(s) and phosphatase(s). This was confirmed by our observation that dCK, activated or not by CdA, can be inactivated in a crude cell extract by purified PP2A. This last result also demonstrates that activation of dCK results from its phosphorylation. We try now to overexpress dCK in HEK 293 or lymphoblastic cells in order to verify if dCK can be effectively labelled with [^{32}P]orthophosphate and if this labelling can be increased by activators of dCK.

Activation of dCK and DNA repair after UV-C irradiation in B-CLL cells

In mammalian cells, various forms of DNA damage elicit a repair response that includes a DNA re-synthesis step which takes place after recognition and removal of the lesions. In resting cells, DNA repair synthesis is usually investigated by measuring incorporation of labelled thymidine into DNA in the presence of hydroxyurea. In a first study, we had investigated the effect of CdA on DNA repair elicited by UV-C irradiation. We observed that CdA inhibits DNA repair synthesis in B-CLL lymphocytes and was able to potentiate UV- C cytotoxicity (9). We also found that the incorporation of deoxycytidine into DNA was much more stimulated by UV-C irradiation than that of dThd. This led us to investigate the effect of UV-C on deoxynucleoside phosphorylating enzymes. The activity of dCK was significantly increased, up to 2.6-fold, 30 min after irradiation with 30 J/m^2 of UV-C, whereas thymidine kinase (TK) activity was not augmented. Activation of dCK by UV-C light was caused neither by a change in the concentration of a low molecular weight metabolite, nor by an increase of the amount of dCK protein, suggesting that it results, like dCK activation by CdA, from a post-translational modification of the enzyme. Preincubation of the cells with the growth factor receptor inhibitor suramin, prevented activation of dCK by UV-C. This polysulfonated drug also completely suppressed not only the increase of the incorporation of dCyd into DNA elicited by UV-C irradiation, but also that of dThd. Our results strongly suggest that both activation of dCK and upregulation of DNA repair synthesis by UV-C are mediated by a membrane-activated signal transduction pathway. We propose that activation of dCK, initiated at the membrane level, could be the prerequisite to DNA repair synthesis induced by UV-C irradiation in B-CLL lymphocytes (10).

Drug sensitivity profiles

With standard treatments of B-CLL, clinical response is variable, depending on drug and patient. To tailor therapy on a more individual basis, we develop an *in vitro* assay which evaluates the sensitivity of the patients' lymphocytes to various drugs (nucleoside analogues, alkylating agents, steroids, anthracyclin, etc.). The lymphocytes will also be characterised by conventional cytogenetics, hybridisation *in situ* (FISH), and molecular biology techniques. By revealing correlations

between chemoresistance profiles and genetic anomalies, these studies might also allow identification of mechanisms of resistance.

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TISSUE-SPECIFIC TRANSCRIPTION FACTORS IN DEVELOPMENT

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*We are studying the role of the **Onecut (OC) transcription factors** in tissue-specific gene expression and development. These factors, discovered in the laboratory, define a new class (1) of conserved homeoproteins, with three members in mammals : HNF-6 or OC-1, OC-2 and OC-3. They display a restricted tissue distribution and the classes of genes that they regulate overlap, but do not superimpose. HNF-6 (**Hepatocyte Nuclear Factor-6**), the OC prototype, was identified (2) as a factor that controls the liver-specific transcription of PFK2/FBPase2. HNF-6 stimulates transcription by at least two different mechanisms depending on the nucleotide sequence of the DNA binding site (3). By interacting with DNA-bound glucocorticoid receptor, HNF-6 can also inhibit glucocorticoid-induced gene transcription in a target-specific way. In adult liver, HNF-6 stimulates transcription of genes coding for enzymes of glucose metabolism, for P450 cytochromes and for secreted proteins. In the embryo, HNF-6 is expressed in the liver and pancreas where it regulates genes coding for other transcription factors. The role of HNF-6 in development has been addressed by studying Hnf6 knockout mice generated in our laboratory. The aim of our current work is to determine the mechanisms by which the OC transcription factors control development of the pancreas, liver and gastrointestinal tract, and to investigate the role of OC factors in diseases of these organs.*

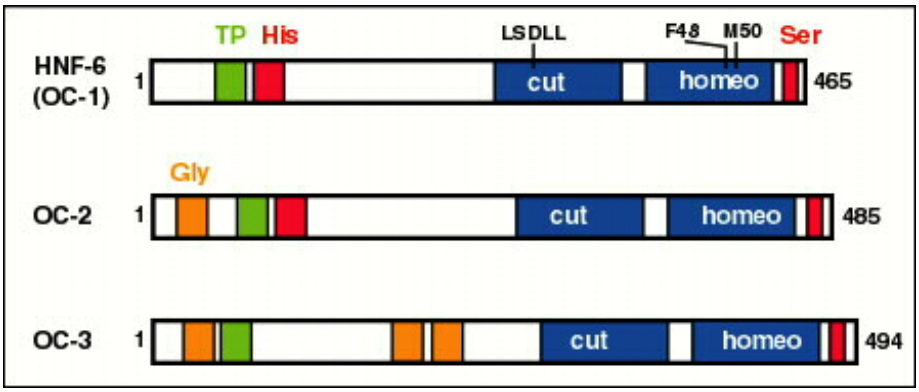

Fig. 1. Stucture of human Onecut proteins. The DNA-binding domain consists of a cut domain and a homeodomain. The cut domain contains a LXXLL motif (LSDLL) that contacts transcriptional co-activators. The homeodomain diverges from other homeodomains by the presence of a phenylalanine at position 48 and a methionine at position 50. These two amino acids are important for recruitment of co-activators. The TP, His, Gly and Ser domains are rich in Threonine/Proline, Histidine, Glycine and Serine, respectively. The TP domain is involved in transcriptional activation, the His and Ser domains in transcriptional repression.

Control and molecular mode of action of HNF-6

Nicolas Plumb, Aurélie Poll, and Jean-Bernard Beaudry

OC proteins contain a divergent homeodomain in which residue 48 is a Phe instead of the canonical Trp and residue 50 is a Met, an amino acid never found at this conserved position in classical homeodomains. They also contain a single cut domain, as opposed to the Drosophila cut protein and its mammalian homologues, which have a classical homeodomain and three cut domains. In addition to the homeo and cut domains, which bind DNA on the sequence DRTCMATND, OC proteins contain other OC-specific conserved regions that modulate their activity (Fig. 1).The Hnf6 gene is made of three exons separated by more than 10 kb. The cut domain is coded by exon 1, and the homeodomain by exon 3. Exon 2 (78 bp) codes for a spacer between these two DNA-binding domains. Differential splicing yields two isoforms with distinct DNA-binding properties, HNF-6α (no spacer) and HNF-6β (with spacer) (1). The study of the rat Hnf6 gene regulatory sequences suggested that it contains more than one promoter. We showed that the liver promoter is a target of STAT5 and in this way HNF-6 plays a role in the effects of growth hormone in the liver. Our current objective is to identify the promoter and regulatory sequences that are active in the pancreas and liver, and the transcription factors that bind to it. These regulatory sequences will be cloned upstream of the β-gal reporter gene and their tissue-specific activity tested in transgenic mice.

By stimulating transcription of the Hnf1β, Hnf4 and Hnf3β genes and by being itself controlled by C/EBPα, HNF-6 participates to the network of liver transcription factors. We are further studying this network, as it now appears that HNF-6 also controls Hnf3α gene expression.

Stimulation of gene transcription by HNF-6 involves co-activators and depends on the integrity of the N-terminal region of HNF-6 (3). We are characterising the transcriptional partners of HNF-6 that are recruited by this region in view of investigating their role in the different spatio-temporal effects of HNF-6 on cell differentiation.

Control of pancreas development by HNF-6

Patrick Jacquemin, Christophe Pierreux, Jonathan van Eyll, Nathalie Houard

Hnf6 knockout mice have an hypoplastic pancreas devoid of islets of Langerhans (Fig. 2). We have shown that HNF-6 is required for the differentiation of pluripotent prepancreatic cells into endocrine precursors. This involves the stimulation by HNF-6 of the proendocrine transcription factor NGN-3 (4). At birth, the knockout mice have too few pancreatic endocrine cells and they become diabetic. We have now found that this glucose intolerance also results from a defective liver expression of the glucokinase gene, which is a target of HNF-6 (5). Type II diabetic patients were screened for mutations in the Hnf6 gene, but none has been identified so far.

Embryonic stem cells, when grown as embryoid bodies, spontaneously generate insulin-producing cells that could be used in therapy of diabetes mellitus. Does differentiation of cells in embryoid bodies mimic that of pancreatic β cells in embryos ? To address this question we verified if the differentiation of the insulin-producing cells in embryoid bodies requires HNF-6. No difference was observed in the expression of insulin between wild-type and Hnf6-/- embryoid bodies. In both cases insulin was expressed in the outer layer of cells, which is similar to the visceral endoderm. In wild-type embryoid bodies HNF-6 was transiently expressed in the outer layer of cells, but was not co-expressed with insulin. The expression of genes that are targets of HNF-6 in developing pancreas was unaffected in Hnf6-/- embryoid bodies. Thus, the differentiation mechanism of insulin-producing cells in embryoid bodies differs from that of the β cells and it is likely to resemble that of insulin-producing cells in the visceral endoderm (6).

As to the pancreas hypoplasy of Hnf6 knockout mice, our data point to a decrease in the number of endodermal precursors specified to a pancreatic fate by the transcription factor Pdx-1. Indeed, in the foetal foregut endoderm, where it is expressed, HNF-6 initiates the expression of Pdx-1, thereby controlling the timing of pancreas specification (7). (Fig. 3)

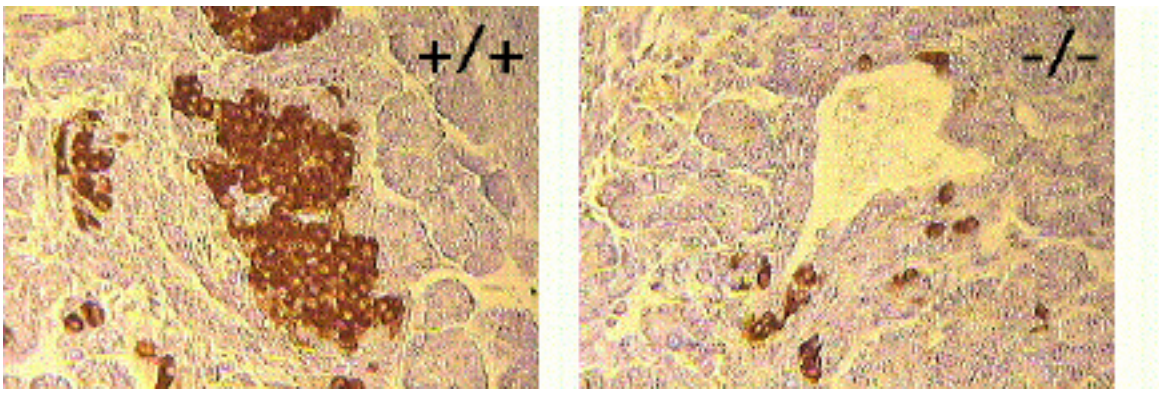

Fig. 2. Abnormal development of the endocrine pancreas in HNF-6 knockout mice. Immunohistochemistry of tissue sections four days after birth shows that only a few insulin-producing cells (brown) are found near pancreatic ducts in Hnf6-/- (right panel), instead of being organized in islets as in Hnf6 +/+ littermates (left panel).

We are now studying the *ex vivo* development of pancreatic rudiments obtained from normal or knockout mouse embryos, in view of rescuing the Hnf6-/- phenotype by candidate gene products identified by microarray analysis.

Control of liver development by HNF-6

Frédéric Clotman, Caroline Bouzin, Nicolas Plumb

During liver development, hepatoblasts differentiate into hepatocytes or into biliary epithelial cells (BEC) which delineate the intrahepatic and extrahepatic bile ducts and the gallbladder. We have shown that HNF-6 is expressed not only in hepatoblasts, but also in BEC. In Hnf6-/- mice, the gallbladder does not develop and the extrahepatic bile ducts are abnormal. The mice suffer from cholestasis and display a phenotype that resembles human biliary diseases called "ductal plate malformations", which are related to biliary atresia. Indeed, the differentiation and morphogenesis of the intrahepatic bile ducts are perturbed (Fig. 4). These disorders involve the transcription factor HNF-1β, which we showed to be a target of HNF-6 and whose expression is downregulated in Hnf6-/- foetal liver (8).

We are further studying the mechanisms of this biliary phenotype by microarray analysis and by exploiting an ex vivo model, the immortalised bipotential mouse embryonic liver (BMEL) cells, which can differentiate into either BEC or hepatocytes.

The developing liver harbours haematopoietic precursors which differentiate into the different blood cell lineages in response to signals sent by hepatoblasts. We have discovered a severe B lymphopenia in young Hnf6-/- mice and have shown that this results from a liver defect, thereby identifying HNF-6 as the first non cell-intrinsic transcription factor known to control B lymphopoiesis specifically in foetal liver.

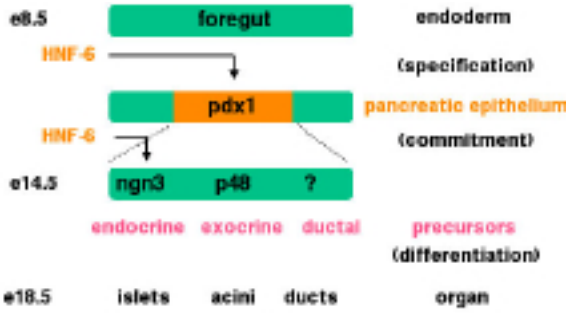

Fig. 3. Role of HNF-6 in pancreas development. In the mouse, pancreas starts developing at embryonic day (e) 8.5 in the foregut endoderm, which becomes specified to a pancreatic fate by the expression of the transcription factor Pdx-1. Cells in this epithelium become precursors that are committed to a ductal, exocrine or endocrine fate. These precursors differentiate into ducts, acini, or the four types of hormone-producing cells which form the islets of Langerhans just before birth. Our analysis of Hnf6-/- embryos shows that HNF-6 controls the timing of pancreas specification by inducing Pdx-1 in the endoderm and that, later on, HNF-6 regulates the development of the endocrine lineage by controlling the transcription factor Ngn3.

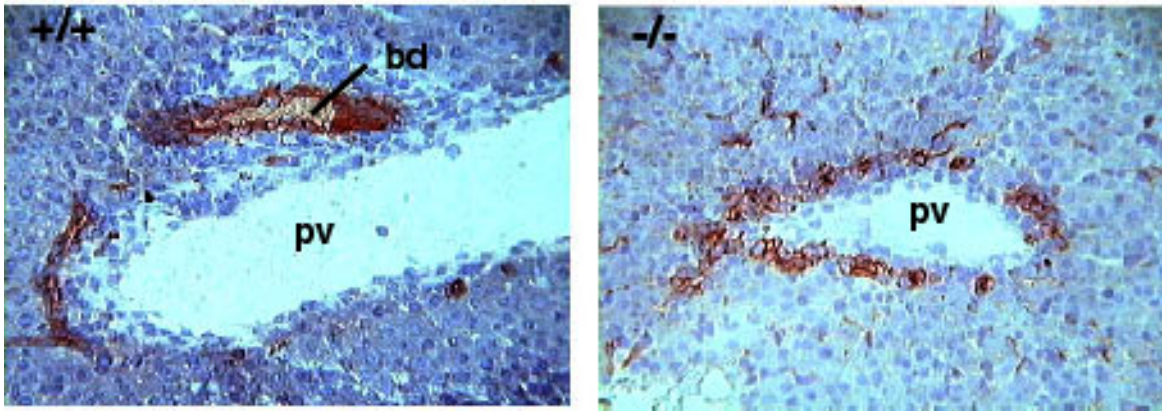

Fig. 4. Abnormal development of the intrahepatic bile ducts in HNF-6 knockout mice.
Immunohistochemistry of liver sections 10 days after birth shows in normal mice (left panel) a typical bile duct (bd) delineated by biliary cells (stained red) embedded in mesenchyme. In contrast, biliary cells do not form bile ducts in Hnf6-/- mice (right panel) and remain dispersed as a layer around a branch of the portal vein (pv).

Role of OC-2 and OC-3 in the development of endodermal derivatives

Patrick Jacquemin, Vinciane Vanhorenbeeck, Christophe Pierreux

We have cloned OC-2 (9) and OC-3 (10) and have shown they have properties similar to those of HNF-6 in terms of DNA-binding and transcriptional activation. In the adult mouse, the patterns of expression of these paralogues are tissue-restricted and partially overlapping with those of HNF-6 (10). While they are all expressed in brain and foregut endoderm, HNF-6 is also expressed in the testis, liver and pancreas, OC-2 in the liver, gut and stomach, and OC-3 in the gut and stomach. In addition, OC-2 is expressed in skin melanocytes where it controls differentiation by regulating the microphthalmia-associated transcription factor (MITF) gene. We have now generated OC-2 and OC-3 knockout mice and are studying their phenotype.

As the three mammalian Onecut genes are expressed in the foregut endoderm, we are setting up cultures of this tissue from normal or knockout mouse embryos in view of exploring *ex vivo* the respective roles of the Onecut factors in pancreas, liver, stomach and gut development.

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and acts upstream of Pdx-1 in the specification cascade. **Dev Biol** 2003, in press.

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SIGNAL TRANSDUCTION AND PROTEIN PHOSPHORYLATION

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Our research concerns the role of protein phosphorylation in the control of metabolism by nutrients, hormones and various stresses. As a model system, we 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 stimulator of glycolysis. Fructose 2,6-bisphosphate was discovered in this Institute by Van Schaftingen, Hue and Hers in 1980 and is the most potent stimulator of 6-phosphofructo-1-kinase (PFK-1), a key enzyme of glycolysis. Fructose 2,6-bisphosphate is synthesised from fructose 6-phosphate and ATP by 6-phosphofructo-2-kinase (PFK-2). Its hydrolysis to fructose 6-phosphate and Pi is catalysed by FBPase-2. These two activities are catalysed at separate sites of a bifunctional enzyme (PFK-2/FBPase-2) 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. We have characterised several PFK-2/FBPase-2 isoforms in mammalian tissues. We also cloned the corresponding mRNAs and showed that they originate from at least two genes (1). These isoforms differ in PFK-2/FBPase-2 activity ratio, kinetic properties and response to phosphorylation by protein kinases. The C-terminus of the heart (H) isozyme, contains phosphorylation sites for several protein kinases. These sites are not present in the other isozymes, such as the liver (L) isozyme, which, by contrast, contains a single phosphorylation site for the cyclic AMP-dependent protein kinase (PKA) at the N-terminus. The concentration of fructose 2,6-bisphosphate changes in response to metabolites, hormones, growth factors, and oncogene activation (1). Over recent years, we made a detailed study of the molecular mechanisms responsible for the activation of heart PFK-2 by insulin and ischemia. This led to the identification of new components of the insulin signalling cascade and to a new interpretation of the Pasteur effect.

Insulin signalling

C. Beauloye, L. Bertrand, U. Krause, V. Mouton, D. Vertommen, L. Hue, M.H. Rider, in collaboration with C. Erneux and S. Schurmans, ULB, Brussels, and D. Alessi, Dundee

SHIP2

SHIP2 (Type II SH2-domain containing inositol 5-phosphatase) antagonises insulin action by hydrolysing phosphatidylinositol 3,4,5-trisphosphate (PIP3) the intracellular second messenger of insulin. We studied mice lacking the SHIP2 gene and found that loss of SHIP2 increased the sensitivity towards insulin, which was characterised by an increased glucose transport and glycogen synthesis in skeletal muscles. This indicates that SHIP2 is a potent negative regulator of insulin signalling and insulin sensitivity (2).

Inhibition of insulin signalling in the ischemic heart

We found that ischemia antagonised insulin signalling in perfused rat hearts. This inhibition resulted from intracellular acidosis, which is a characteristic feature of ischemia. Ischemic acidosis was found to decrease the kinase activity and tyrosine phosphorylation of the insulin receptor, thereby preventing activation of the downstream components of the signalling pathway (3).

Activation of heart PFK-2 by insulin

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. The mechanism involved in this insulin-induced activation of heart PFK-2 is being studied both *in vitro* and in intact cells. The recombinant heart isozyme of PFK-2 is a substrate of several protein kinases, and especially of protein kinases of the insulin signalling pathways, such as protein kinase B or Akt, which is believed to mediate most metabolic effects of insulin (4). We tested the role of PKB in the activation of PFK-2 by insulin using a 'dominant-negative' construct and found that the activation of PFK-2 by insulin did not require PKB, but was mediated by another protein kinase located downstream of PDK1 (5). We purified a wortmannin-sensitive and insulin-stimulated protein kinase (WISK). WISK phosphorylates heart PFK-2 on Ser466 leading to its activation. Our current efforts are aimed at identifying and cloning this protein kinase, which differs from known protein kinases of the insulin signalling pathways (6).

AMP-activated protein kinase

C. Beauloye, L. Bertrand, C. Bouzin, S. Horman, U. Krause, A.-S. Marsin, , D. Meisse, D. Vertommen, M.H. Rider, L. Hue, in collaboration with J.-L. Vanoverschelde, UCL, G. Van den Berghe and M.-F. Vincent, ICP, Brussels, M. Van de Casteele and D. Pipeleers, VUB, Brussels, D. Carling and A. Woods, London, D.G. Hardie and C. Proud, Dundee, R. Bartrons, Barcelona, P. Ferré and F. Foufelle, Paris, A. Lavoinne, Rouen and T. Walliman, U. Schlattner and D. Neumann, Zurich

The AMP-activated protein kinase (AMPK) acts as a fuel gauge in the cell. AMPK is activated by an increase in the AMP/ATP ratio as it occurs when the oxygen supply is restricted or after exposure of cells to inhibitors of the mitochondrial respiratory chain, such as oligomycin. In certain cells, AMPK can also be activated by AICA-riboside, which enters cells to be phosphorylated into ZMP, an analogue of AMP. AMPK switches off energy-consuming biosynthetic pathways, thereby conserving ATP.

Stimulation of heart glycolysis by ischemia.

Ischemia or anoxia stimulates glycolysis (Pasteur Effect) which involves increased glucose transport and PFK-2 activation in heart. We investigated whether AMPK could mediate this phenomenon. AMPK phosphorylated heart PFK-2 on Ser 466 which led to its activation. In perfused hearts, ischemia induced an activation of AMPK, which correlated with PFK-2 activation and with an increase in Fru-2,6-P2 concentration. In cultured HEK-293 cells transfected with heart PFK-2, a dominant negative construct of AMPK abolished both the phosphorylation and activation of transfected PFK-2 induced by oligomycin, an inhibitor of oxidative phosphorylation. Therefore, heart PFK-2 is a new substrate of AMPK and its activation is involved in the Pasteur Effect (7).

Inducible PFK-2

A novel isoform of PFK-2, which is induced by pro-inflammatory stimuli and therefore called inducible PFK-2 (iPFK2), is expressed constitutively in several human cancer lines. In monocytes, iPFK-2 is induced by lipopolysaccharide (LPS), a component of the

outer membrane of gram-negative bacteria, which triggers an inflammatory response. iPFK-2 resembles the heart isozyme in that it contains a serine residue (Ser 461) in a similar context to Ser 466 of heart PFK-2 for phosphorylation by AMPK. Recombinant iPFK-2 was indeed phosphorylated and activated by AMPK *in vitro*. In cultured human monocytes activated by LPS, hypoxia activated AMPK and iPFK2. This activation correlated with an increase in the concentration of fructose 2,6-bisphosphate and with a stimulation of the glycolytic flux. In cultured HEK-293 cells, a dominant-negative construct of AMPK abolished the activation of transfected iPFK2 by oligomycin. We propose that the stimulation of monocyte glycolysis via the AMPK-induced phosphorylation and activation of iPFK-2 could be important for furnishing ATP to sustain cytokine synthesis in infected anaerobic tissues (8).

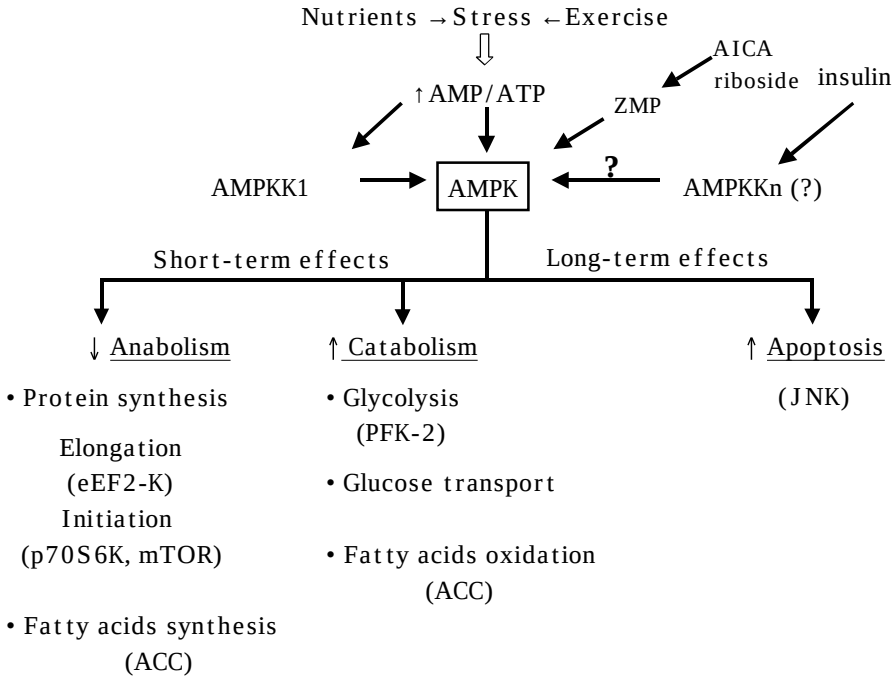

Fig. 1. Mechanism of activation and targets of AMP-activated protein kinase (AMPK). AMPK is phosphorylated and activated by AMPKK when the AMP/ATP ratio increases as a result of metabolic stresses. It can also be activated by an AMP analogue, ZMP, which is formed from AICA-riboside. Insulin antagonises AMPK activation by a still unknown mechanism. The targets of AMPK, which we discovered and which are responsible for some of its short-and long-term effects, are indicated. AMP, adenosine monophosphate; ATP, adenosine triphosphate; AICA riboside, 5-aminoimidazole-4-carboxamide riboside; ZMP, AICA ribotide; AMPK, AMP-activated protein kinase, AMPKK, AMPK-kinase; eEF2K, eukaryotic elongation factor-2-kinase; p70S6K, p70 ribosomal protein S6 kinase; mTOR, mammalian target of rapamycin; ACC, acetyl-CoA carboxylase; PFK-2, 6-phosphofructo-2-kinase; JNK, c-jun kinase

AMPK activation inhibits protein synthesis

Protein synthesis, in particular peptide chain elongation, consumes a large proportion of intracellular ATP. Therefore, we investigated whether AMPK activation could inhibit protein synthesis via the phosphorylation of regulatory components of the translation machinery. In anoxic rat hepatocytes or in hepatocytes treated with 5-aminoimidazole-4-carboxamide (AICA) riboside, AMPK was activated and protein synthesis was inhibited. The inhibition of protein synthesis could not be explained by changes in the phosphorylation states of factors known to control the initiation of translation, such as factor 4E-binding protein-1 or eukaryotic initiation factor 2a. However, the phosphorylation state of eukaryotic elongation factor 2 (eEF2) was increased in anoxic hepatocytes. In HEK-293 cells, transfection of a dominant negative AMPK construct abolished the oligomycin-induced inhibition of protein synthesis and eEF2 phosphorylation. Lastly, eEF2 kinase, the kinase that phosphorylates eEF2, was activated in anoxic or AICA riboside-treated hepatocytes. Therefore, the activation of eEF2 kinase by AMPK, resulting in the phosphorylation and inactivation of eEF2, provides a novel mechanism for the inhibition of protein synthesis (9).

Incubation of hepatocytes with amino acids, such as glutamine and leucine, leads to an activation of biosynthetic pathways, such as glycogen synthesis, lipogenesis and protein synthesis. Under these conditions, p70 ribosomal S6 kinase (p70S6K), a protein kinase that participates in the control of protein synthesis and is activated in response to hormones, mitogens and nutrients, becomes activated via activation of the mammalian target of rapamycin (mTOR) by an unknown mechanism. Pretreatment of hepatocytes with AICA riboside prevented the activation and phosphorylation of p70S6K. Therefore, it is likely that AMPK inhibits p70S6K activation by phosphorylating target(s) in the mTOR signalling pathway.

Sustained activation of AMPK triggers apoptosis in liver cells

We studied the effect of long-term AMPK activation on liver cell survival. AMPK activation was maintained in FTO2B cells treated with AICA riboside or by adenoviral transfection of hepatocytes with constitutively active AMPK. Sustained AMPK activation triggered apoptosis through an activation pathway involving c-Jun kinase and caspase-3 (10).

AMPK activation by upstream kinases

AMPK activation requires phosphorylation in the activation loop at Thr172 of its catalytic alpha-subunit by an upstream kinase, AMPK-kinase, itself sensitive to AMP. We studied the effect of insulin on this phenomenon. We observed that AMPK activation in hypoxic hearts was antagonised by a pre-treatment of the hearts with insulin. The effect of insulin was blocked by wortmannin, an inhibitor of PI 3-kinase and resulted in a decreased phosphorylation state of Thr172 in AMPK. In addition, the insulin effect was unrelated to changes in the AMP/ATP ratio, thus demonstrating that AMPK activity could be modified by a mechanism independent of the AMP/ATP ratio in cardiomyocytes.

As stated above, evidence is growing that other kinases (AMPKKs), which are not AMP-sensitive, are also involved in the control of AMPK activity. Using partially purified AMPKK to phosphorylate bacterially expressed AMPK heterotrimers, we have identified upstream kinase phosphorylation sites in AMPK by mass spectrometry (see below). In addition to confirming the phosphorylation of Thr 172, new sites have been identified in the α-1 and α -2 catalytic subunits. The role of these sites is being studied by site-directed mutagenesis and transfection. The AMPKKs are being purified from heart and liver for characterisation.

Stimulation of heart glycolysis by increased work

In skeletal muscle, contraction activates AMPK and stimulates glycolysis by promoting glucose transport. Therefore, we investigated whether submitting hearts to an increased workload would stimulate glycolysis via the AMPK-induced activation of PFK-2. Increasing the workload indeed activated PFK-2, but AMPK activity was unchanged. The PI 3-kinase inhibitor, wortmannin, counteracted the workload-induced stimulation of glycolysis and PFK-2 activation. Moreover, PKB was activated by increasing the workload and this effect was abrogated by wortmannin. We conclude that increasing heart work stimulates glycolysis through the PI 3-kinase/PKB or WISK signalling pathway independently of AMPK. This contrasts with the situation in contracting skeletal muscle and ischemic heart, where the stimulation of glycolysis involves AMPK activation.

Control of smooth muscle contraction

D. Vertommen and M.H. Rider in collaboration with P. Gailly, UCL, D. Carling, London, and M. Walsh, Calgary, Canada

In earlier work, we 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 (PKD). However, there are no

physiological substrates yet recognised for this protein kinase. We have shown that in various smooth muscles stimulated with vasoconstrictors, such as vasopressin, PKD becomes activated. Moreover, we have discovered new in vitro substrates for PKD in the contractile machinery that could be implicated in the prolonged phase of muscle contraction when Ca^{2+}-dependent P-light chain phosphorylation of myosin is not involved in force generation. We are currently looking at the agonist-induced development of force in femoral artery strips to see whether, using mass spectrometry, the phosphorylation sites on proteins phosphorylated by PKD *in vitro* are phosphorylated in response to agonists *in vivo*. This work has potential for the understanding of hypertension and could lead to new treatments for this condition.

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ENDOCYTOSIS

Pierre J. COURTOY, Member (right)
Marie-France VANDENBROUCKE, Member (left)

Idesbald COLIN, Guest Investigator
Patrick VAN DER SMISSEN, Assistant Member
Karine CROIZET, Philippe Delori Postdoc Fellow
Philippe de DIESBACH, Postdoctoral Fellow
Marcel METTLEN, Graduate Student
Anna PLATEK, Graduate Student
Geneviève DOM, Research Associate
Marianne EPPE, Technician
Thanh LAC, Technician (half-time),
Michèle LERUTH, Technician
Olga MEERT, Technician (half-time)
Francisca N'KULI, Technician
Yves MARCHAND, Secretary

Endocytosis is a central activity of all eukaryotic cells, that allows for cell nutrition, regulates the composition of the cell surface and controls transfer of macromolecules across epithelial barriers. The role of endocytosis in signalling is also increasingly recognized. This research group has made significant contributions in the dissection of endocytic pathways (1) and its contribution to physiopathology (2), parasitology (3,4) as well as pharmacology (5,6), and is currently unravelling the molecular machineries controlling the endocytic activity at the apical surface of epithelia and upon malignant transformation. Recent achievements include the elucidation of the signalling cascade whereby the paradigmatic oncogenes, v-Src and K-Ras, control the actin cytocortex (7,8); the role of apical endocytosis in the regulated production of thyroid hormones (9); and the elucidation of a deficit of apical endocytosis in a genetic form of kidney stones (10).

Oncogene-induced macropinocytosis in fibroblasts

M. Amyere, M. Mettlen, P. Van Der Smissen and P.J. Courtoy

We recently reported that v-Src and K-Ras caused a profound remodelling of actin cytoskeleton in Rat-1 fibroblasts, resulting in stress fibre disappearance, cortical actin polymerisation, ruffling and macropinocytosis (8). These alterations were found to depend on the constitutive sequential activation of phosphoinositide 3-kinase (PI3K) and phospholipase C (PLC). Noticeably, there was no effect of v-Src, K-Ras and overall activation of PI3K and PLC on the receptor-mediated endocytosis of transferrin by fibroblasts, underscoring the difference in the molecular machinery supporting micropinocytosis via clathrin-coated pits (i.e. receptor-mediated endocytosis of transferrin) and macropinocytosis upon closure of membrane ruffles. Recent investigations also involve phospholipase D in macropinocytosis.

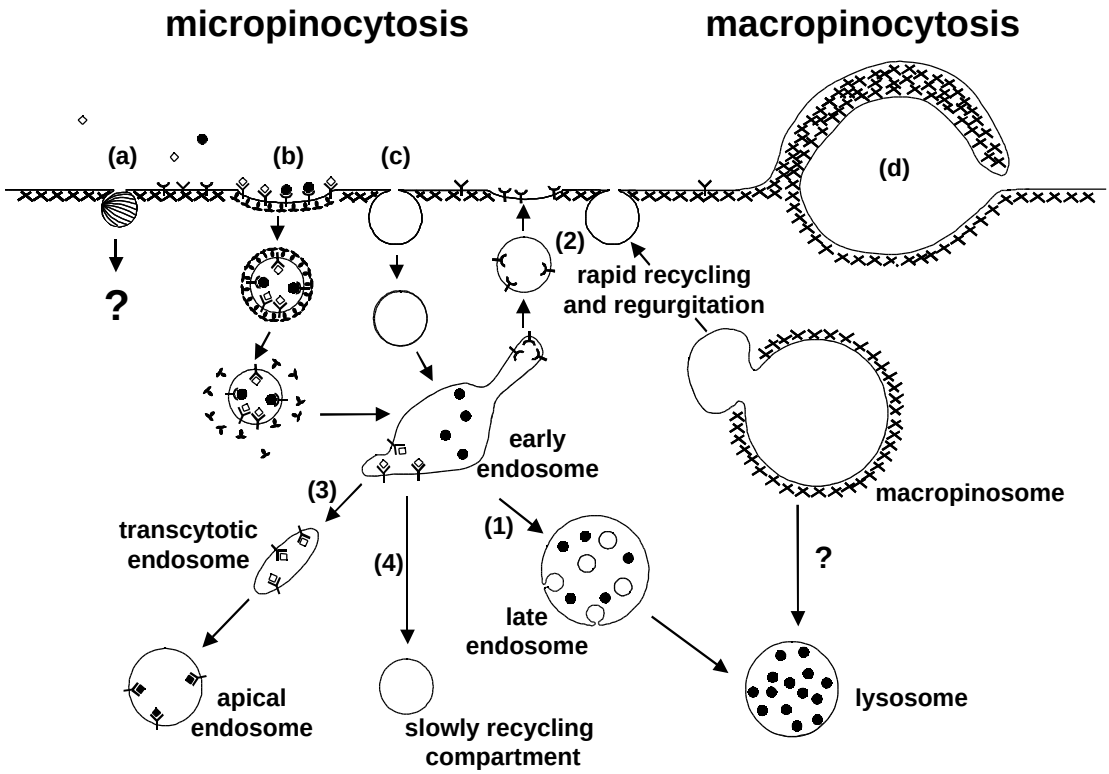

Fig. 1. Pathways of endocytosis. This scheme represents four possible modes of vesicular entry of solutes into cells : (a) caveolae; (b) clathrin- and dynamin-associated pits, or "coated pits"; (c) clathrin- and dynamin-independent micropinocytic pits; and (d) macropinocytosis. Crosses represent cortical 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 slowly recycling endosomes.

Regulation of endocytosis by v-Src in polarized cells

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

Since most cancers are of epithelial origin, and since apical endocytosis depends on actin, we examined whether v-Src would similarly trigger fluid-phase endocytosis in MDCK cells and whether apical endocytosis would be selectively affected. However, because transformation causes a rapid loss of epithelial polarity, we resorted to MDCK cells bearing a thermosensitive v-Src kinase (MDCK/tsLA31 cell line). In their non-polarized state, shifting from the non-permissive temperature (40°C) to the permissive temperature for v-Src kinase (34°C) accelerated fluid-phase endocytosis in a PI3K- and PLC-dependent manner, as shown by inhibition by wortmannin and NCDC, respectively. When MDCK/tsLA31 cells were plated at high density on a permeable support and cultured at 40°C, a polarized epithelial monolayer could be established, with closely apposed tight junctions, and ~ 90 % of transferrin-receptors being exposed at the basolateral surface. This epithelium showed a 2-fold faster rate of fluid-phase endocytosis at the basolateral than at the apical surface, and a similar rate of receptor-mediated endocytosis of transferrin at both membrane domains. Upon shift to 34°C for 6 h, whereas the rate of fluid-phase endocytosis at the basolateral surface was not affected, there was a dramatic (up to 6-fold) increase of fluid-phase endocytosis at the apical surface. This acceleration was dependent on PI3K and PLC. These data indicate that v-Src selectively affects the apical plasma membrane, regarded by some investigators as the equivalent of a large raft.

Relation between endocytosis and cell motility

A. Platek, M. Mettlen and P.J. Courtoy

It has been proposed that endocytosis contributes to cell motility by allowing for selective removal of plasma membrane constituents from the trailing edge and their recycling to the leading edge. Since v-Src accelerates both motility and endocytosis, we examine whether these two processes are linked and

depend on the same regulatory machinery. To this aim, we made use of fibroblasts and MDCK cells harbouring thermosensitive v-Src kinase (Rat-1/tsLA29 and MDCK/tsLA31 cells). In both cell lines, v-Src activation led to a 2-fold acceleration of cell motility, as evidenced by the population-based wound healing assay and by single cell recording in Dunn chambers. Accelerated motility was selectively abrogated by PI3K, PLC and PLD inhibitors. These observations suggest a link between accelerated motility and endocytosis.

Regulation of transcytosis

P.J. Courtoy, in collaboration with J.P. Vaerman and D. Giffroy, MEXP

We have continued our long-lasting collaboration with Dr. J.P. Vaerman to address the regulation of transcytosis in transfected MDCK cells bearing the rabbit polymeric Ig-receptor. Receptor occupancy by polymeric IgA, which accelerates transcytosis, was found to activate PLC, as shown by an increased level of inositol 3,4,5-trisphosphate. In the absence of added polymeric IgA, transcytosis of polymeric Ig-receptor was also accelerated by the co-operative effect of the two downstream arms of the PLC response, i.e. by increasing cytosolic calcium concentration with ionomycin, and activation of protein kinase C via phorbol esters. Thus, PLC is involved in signalling, not only of v-Src-induced apical endocytosis (see above), but also in a presumably late step of basolateral-to-apical transcytosis of the polymeric Ig-receptor. It is likely that both effects are mediated by the actin cytoskeleton.

In collaboration with Dr. R. Fuchs and her colleagues (Vienna, Austria), we have further dissected the sequential endosomal compartments along the transcytotic route, using the isolated perfused rat liver system, selective temperature shifts and exploiting the resolution of free-flow electrophoresis.

Endocytosis regulates thyroid hormone production

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

The production of thyroid hormones results from endocytosis by thyrocytes of thyroglobulin stored in the colloid, followed by intracellular proteolysis. Since both substrates and hydrolytic enzymes are in vast excess, we hypothesised that the production of thyroid hormones is regulated by their encounter, i.e. depends on rate-limiting endocytic catalysts. To test this hypothesis, we have followed two approaches. First, we found that the increased level of expression of the rate-limiting endocytic catalysts, Rab5a and Rab7, in autonomous hyperactive adenomas, closely correlates with (i) a decrease in residual thyroglobulin content; and (ii) an increased recovery of particulate iodine towards more distal compartments (i.e. the most active proteolytic organelles of the degradative pathway). Second, having established polarized human thyrocytes that are competent for selective basolateral delivery of thyroid hormone, we found that TSH stimulation or direct activation of the cAMP cascade are sufficient to increase Rab5a and Rab7 expression (9).

Alterations in the endocytic apparatus of mice lacking renal chloride channel, ClC-5, account for proteinuria in Dent's disease

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

To elucidate the molecular basis of Dent's disease, an X-linked familial form of nephrolithiasis, we have analysed the endocytic apparatus in knockout mice for the kidney-specific chloride channel, ClC-5, by a systematic comparison of the steady-state level of several major constituents and regulators of the apical endocytic apparatus, their immunolocalisation at the ultrastructural level, and the follow-up by analytical subcellular fractionation of an apical endocytic tracer, at various intervals of uptake. These investigations benefit from a close interaction with Dr. W. Guggino (John Hopkins, Baltimore, MD, USA) and a new collaboration launched with Dr. E.I. Christensen (Aarhus, DK). Results indicate that defective ClC-5 in Dent's disease patients and KO mice leads to a major trafficking defect of the low-molecular weight protein receptors, megalin and cubilin (10).

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 potential generic therapeutic approach. However, ODN efficiency is hampered by limited cellular uptake and transmembrane translocation to the cytosol/nucleosol where ODN targets reside. Having identified a candidate ODN receptor on HepG2 cells as a ~66-kDa band, we have re-examined in these cells the kinetics of ODN uptake, subcellular distribution and intracellular localization by confocal microscopy, at concentrations relevant to study a receptor-

dependent process, and in comparison with well-established tracers of fluid-phase and receptor-mediated endocytosis. These data suggested that ODN was internalised at low concentrations into unique endocytic structures that did not contain transferrin, then to dense structures that were distinct from lysosomes.

However, we recently disclosed, to our great surprise, that the ~66-kDa band is actually induced by *Mycoplasma* infection of the HepG2 cell line worldwide. Moreover, this band could be identified as an invariant membrane protein of *Mycoplasma hyorhinis* itself, based on ribosomal DNA sequencing combined with ODN ligand blotting after immunoprecipitation by a monoclonal antibody against the *Mycoplasma* protein. Whereas antibiotic treatment of infected HepG2 cells strongly decreased ODN capture, as measured by a biochemical assay, conversely, deliberate infection of HeLa cells with *Mycoplasma hyorhinis* dramatically promoted ODN uptake but did not affect receptor-mediated endocytosis of transferrin. This was confirmed by confocal microscopy of infected HepG2 cells, that also showed an undistinguishable labelling pattern after exposure of living cells to fluorescent ODN and after immunolabelling of the *Mycoplasma* protein. We propose that ODN binds to the major invariant membrane protein of *Mycoplasma hyorhinis* when still attached at the cell surface, after which the complex is internalised by "piggy-back" endocytosis (5).

Azithromycin, a lysosomotropic antibiotic, impairs pinocytosis in cultured cells

P.J. Courtoy, P. Van Der Smissen and M Mettlen, in collaboration with M.-P. Mingeot, D. Tyteca and their colleagues, FACM

We found that the dicationic macrolide antibiotic, azithromycin, inhibits the uptake of horseradish peroxidase (HRP) by fluid-phase pinocytosis in fibroblasts in a time- and concentration-dependent fashion without affecting its decay (regurgitation and/or degradation). Upon short-term azithromycin treatment, ultrastructural cytochemistry disclosed a decreased labelling by HRP of endosomes (5 min pulse) and no labelling of lysosomes (2 h). Azithromycin also caused the appearance of large, light- and electron-lucent vacuoles, that were labelled by lucifer yellow if this endocytic tracer had been taken up in lysosomes prior to azithromycin treatment, consistent with acidotropic swelling of late endocytic compartments. Long-term (3 days) exposure to azithromycin resulted in the accumulation of very large vesicles filled with pleiomorphic content, consistent with lysosomal phospholipidosis. These vesicles were intensively stained with filipin, indicating a mixed storage with cholesterol. The impairment of HRP pinocytosis directly correlated with the amount of azithromycin accumulated by the cells, but not with the phospholipidosis induced by the drug. The proton ionophore monensin, which suppresses azithromycin accumulation, also prevented inhibition of HRP uptake. We suggest that azithromycin (i) inhibits fluid-phase pinocytosis by impairing the formation of pinocytic vacuoles and endosomes; (ii) causes acidotropic swelling of lysosomes, then a mixed lysosomal storage disorder; and (iii) thereby impairs their accessibility to endocytic tracers (6).

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EXTRACELLULAR MATRIX BREAKDOWN

Pierre J. COURTOY, Member (right)
Yves EECKHOUT, Member (left)
Etienne MARBAIX, Member (middle)

Patrick HENRIET, Member
Hervé EMONARD, Guest Investigator
Christine GALANT, Assistant Member
Alix BERTON, ICP Fellow
Patricia CORNET, Graduate Student
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Vassil VASSILEV, Graduate Student
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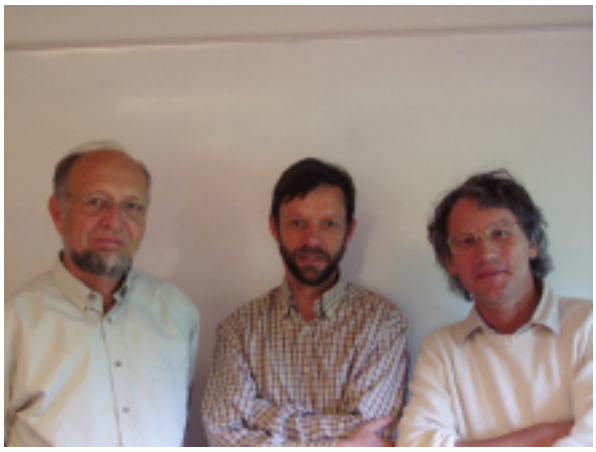
Olga MEERT, Technician
Yves MARCHAND, Secretary



The extracellular matrix (ECM) plays a central role in the structural and functional organization of tissues and organs. ECM constituents, in particular fibrillar collagens, are the most abundant proteins of the human body. Physiological and pathological breakdown of ECM is predominantly achieved by a family of neutral metalloproteinases, called matrix metalloproteinases (MMPs). Our group has a long-standing expertise in the biochemistry and molecular biology of collagenase and related MMPs (1,2). We have demonstrated that menstrual and abnormal uterine bleeding in women are due to the expression and activation of some MMPs (3,4). This seminal observation led us to : (i) exploit this system as a human model to study the regulation of MMPs, in particular cellular interactions that integrate overall hormonal impregnation with local environmental changes (5, 6 and ongoing research programme); and (ii) to explore whether this basic knowledge can lead to a rational treatment of abnormal uterine bleeding..

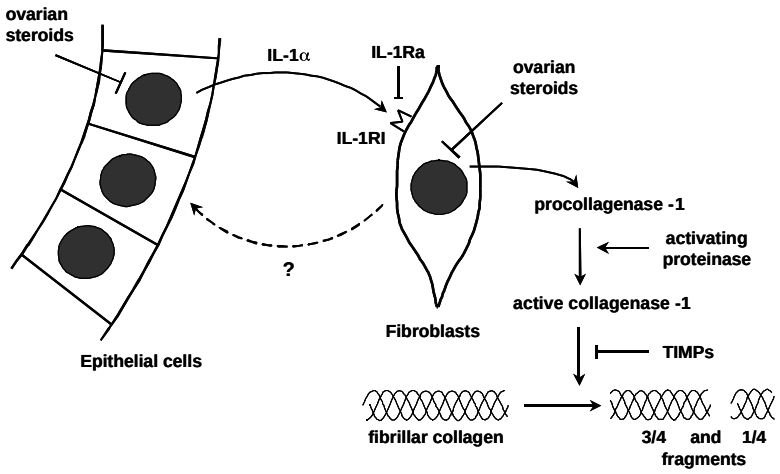


Fig. 1. Model of MMP regulation in the human endometrium : the case of MMP-1. Upon ovarian steroids withdrawal, IL-1_ is released by endometrial epithelial cells and triggers in adjacent fibroblasts the production of proMMPs, including interstitial procollagenase-1. Fibroblast activation involves interleukin-1 receptor (IL-1RI) 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.

Regulation of pro-MMP activation in the human endometrium

V. Rigot, A. Berton, E. Marbaix, P.J. Courtoy and Y. Eeckhout

Most matrix metalloproteinases (MMPs) are secreted as inactive pro-enzymes. Their expression is well documented in several human tissues, but their activators *in vivo* are still unknown. To address this question, the activation of progelatinase B (proMMP-9) in the human endometrium was selected as an example. ProMMP-9 was detected by gelatin-zymography in homogenates of fresh endometrial tissue sampled during all phases of the menstrual cycle, whereas its active form was observed only during the late secretory and menstrual phases. Furthermore, proMMP-9 was expressed and activated in endometrial explants sampled outside the perimenstrual phase when cultured in the absence of both progesterone and oestradiol, i.e. mimicking *in vitro* the menstrual condition. Analysis of such tissue cultures by gelatin zymography and Western blotting showed that activation of proMMP-9 depended on a secreted factor and was selectively inhibited by either a synthetic inhibitor of stromelysin 1 (MMP-3) or a monoclonal antibody that specifically blocks MMP-3, thus providing strong evidence for the activation of proMMP-9 *in vivo* by MMP-3. The activation of proMMP-3 was itself inhibited by a broad range MMP inhibitor in most cultures, but seemed to involve multiple pathways, implying both serine proteinases and metalloproteinases, which could operate in parallel or sequentially. Altogether, these data point to a highly-regulated MMP activation cascade (7).

Regulation of the expression of MMPs, TIMPs and endometrial cytokines

P. Henriet, P. Cornet, C. Picquet, V. Vassilev, P.J. Courtoy, Y . Eeckhout and E. Marbaix

Both endocrine (3,8) and paracrine factors (5,6) participate in controlling the expression and activity of the MMPs involved in the menstrual breakdown of the human endometrium. During this year, our research has been focused on potential regulatory cytokines of the TGF-β family: TGF-β 1 and 2, and the recently discovered LEFTY-A, also called endometrial bleeding associated factor (EBAF), the human orthologue of the murine lefty 1 gene. By competitive RT-PCR and, when possible, immunoassay, we have quantified the endometrial expression of these cytokines along the normal menstrual cycle *in vivo* and in explant cultures. Despite important variations between different endometria, our observations point to a down-regulation of TGF-β2 by ovarian steroids. We have also found that LEFTY-A and TGF-β2 mRNA concentrations were strikingly (100-fold) increased *in vivo* in endometria showing signs of menstrual breakdown. A similar increase was observed in proliferative endometria when cultured for 24 h in the absence of ovarian steroids and was prevented by the addition of progesterone, indicating an hormonal control of the expression of LEFTY-A in the human endometrium (9).

Regulation of gelatinases A and B by long-chain unsaturated fatty acids

H. Emonard, A. Berton, V. Rigot and Y. Eeckhout, in collaboration with CNRS, Reims, France

The matrix metalloproteinases gelatinase A (MMP-2) and gelatinase B (MMP-9) are implicated in the physiological and pathological breakdown of several extracellular matrix proteins. We found that long-chain fatty acids selectively inhibit gelatinases A and B. The inhibition of gelatinases increased with fatty acid chain length and unsaturation. *Ex vivo* experiments on human skin tissue sections have shown that micromolar concentrations of a long-chain unsaturated fatty acid (elaidic acid) protect collagen and elastin fibers against degradation by gelatinases A and B, respectively. The role of the fibronectin-like domain, unique to gelatinases, in long-chain fatty acids binding and protease inhibition was demonstrated using a recombinant fibronectin-like domain of gelatinase A and a mutant of gelatinase A deleted of this domain. Moreover, surface plasmon resonance studies with the three individual type II modules of the fibronectin-like domain of gelatinase A identified the first type II module as primarily responsible for long-chain fatty acids binding.

Role of matrix metalloproteinases in abnormal endometrial bleeding

C. Galant, Y. Eeckhout, P.J. Courtoy and E. Marbaix, in collaboration with J.L. Brun, Bordeaux, France

Since matrix metalloproteinases (MMPs) play a key role in initiating normal menstrual breakdown, we looked for their contribution in triggering excessive, prolonged or irregular bleeding, those functional menstrual disorders that lead to one fourth of hysterectomies. Patients upon progestin-only contraception, a characteristic condition in which irregular bleeding is common, were selected for this investigation and paired biopsies compared at the time of bleeding and during non-bleeding intervals. Irregular bleeding was clearly associated with focal menstrual-like stromal breakdown and increased

expression and activation of several MMPs, together with decreased production of TIMP-1 (10). This association was recently confirmed in all major cases of irregular bleeding, irrespectively of hormonal treatment. An ongoing collaboration with Dr. J.L. Brun addresses whether the same molecular mechanisms account for recurrence of bleeding after selective endometrial resection and thermoablation. To clarify the reason for these local disorders, we currently analyse cytokines that control the expression and activation of MMPs and could be abnormally expressed and/or secreted at the time of irregular bleeding.

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CONNECTIVE TISSUE AND ARTHRITIS

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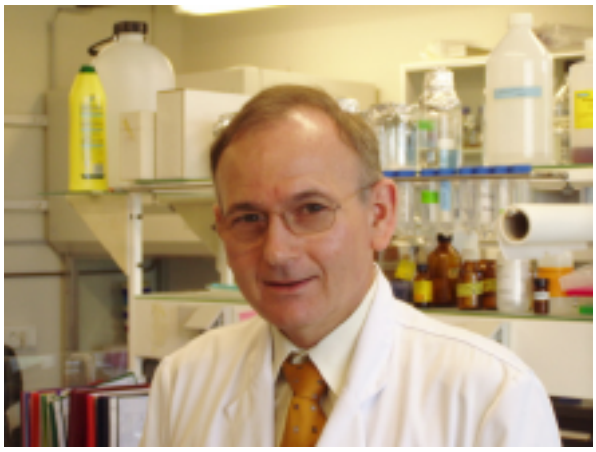

Among the many rheumatic disorders, the osteoarthritic diseases (OA) are the most prevalent disorders of the joint, with radiographic evidence seen in at least 70 % of the population over 65 years of age. OA is a group of overlapping distinct diseases, which may have different etiologies but with similar biologic, morphologic, and clinical outcomes. The disease processes not only affect the articular cartilage but involve the entire joint, including the subchondral bone, ligaments, capsule, synovial membrane, and periarticular muscles.

Effects of nonsteroidal anti-inflammatory drugs on the overall metabolism of articular cartilage

Because they inhibit cyclo-oxygenase (COX), and hence the production of prostaglandins (PGs), non-steroidal anti-inflammatory drugs (NSAIDs) are widely prescribed to patients suffering from arthritis. 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, in addition to their anti-inflammatory properties, have been implicated in the protection of the gastrointestinal tract from injury.

Although NSAIDs undeniably produce relief of pain and improvement of joint mobility in patients suffering from arthritis, *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 organization 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 (1, 2).

We therefore investigated the action of celecoxib (a strong selective COX-2 inhibitor) on the metabolism of newly synthesized HA and proteoglycan molecules in explants from human OA cartilage (3). In contrast to classical NSAIDs, this COX-2 selective inhibitor had a positive effect on the overall metabolism of both proteoglycans and hyaluronan, two major components of the extracellular matrix of cartilage. This effect, which is independent of the inhibition of prostaglandin production, is under investigation as it might be of

great biological and therapeutic significance in arthritis.

Markers of connective tissue metabolism in health and disease

In collaboration with E. Thonar, Rush-Presbyterian-St Luke's Medical Center, Chicago, USA.

Nowadays, several biochemical molecules derived from the joint components can be quantified in body fluids (joint fluid, blood and urine) (4 – 7). Theses molecules termed "metabolic markers" or simply "markers" appear as important tools to disclose *in vivo* important changes occurring during both the preclinical and clinical stages of various joint diseases, including osteoarthritis (6, 7). There is also evidence that these markers may prove helpful in determining whether a therapeutic regimen is effective or not, and this in a relatively short period of time (5, 6). Indeed, in the absence of markers, the efficacy of treatment in joint disorders relies mainly on radiographic changes, an approach that takes years before one can reach meaningful results.

The markers that are most currently used are hyaluronan, a marker of synovial proliferation and inflammation, antigenic keratan sulfate, a marker of proteoglycan metabolism, cartilage oligo-matrix protein, a marker of cartilage matrix remodeling, and the telopeptides of type II collagen, a marker of the breakdown of cartilage collagen (6). There is indeed good agreement that this panel of markers helps diagnose, monitor or prognosticate osteoarthritic changes.

Role of the subchondral bone in the initiation and progression of the osteoarthritic disease process

So far, the possible role of subchondral bone in the initiation and/or progression of osteoarthritis (OA) has focused little investigative attention. We have therefore explored this topic in an animal model of osteoarthritis. In this model, severing of the anterior cruciate ligament of the knee increases dramatically the biomechanical forces applied to the internal compartment of the knee joint and results in the progressive apparition of OA lesions in the operated joint which closely resemble those seen in human OA.

During the first weeks following joint destabilisation, we have observed a dramatic decrease in the density and volume of the trabecular subchondral bone. These changes increased with time post-surgery and were restricted to the internal compartment of the operated joint whereas no significant changes in bone density and volume could be disclosed in the external compartment of the unstable joint.

Obviously, theses changes reflect an adaptation of the bone to absorb the enhanced biomechanical forces imposed upon it. On the other hand, these changes concomitantly induce a dramatic increase in the tensile and shearing forces upon the overlying articular cartilage and, in so doing, contribute to the degradation of the cartilage tissue. Our working hypothesis is supported by the finding that animals receiving drugs known to inhibit bone resorption do not show up any change in the volume and density of the trabecular subchondral bone of the operated knee and, more importantly, exhibit a dramatic decrease in the severity of cartilage OA lesions (8).

These findings open a new approach in the therapeutic regimen of OA and studies are currently conducted in human OA. Results of the preliminary clinical trial will be known during the year 2003.

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 the 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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METABOLIC COMPARTMENTATION IN TRYPANOSOMES

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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 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 (1). 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 closely related parasites such as* Trypanosoma cruzi *and* Leishmania mexicana, *and use the collected information for the design of effective and selective inhibitors by structure-based and catalytic mechanism-based approaches (2).*

In addition our research aims at understanding what controls 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 both insight into the consequences of the compartmentation of the pathway and information as to which enzymes of the pathway are the best targets for drugs.*

Several enzymes of another pathway of carbohydrate metabolism: the hexose monophosphate pathway, involved in the generation of intermediates essential for cell growth, cell division and protection against oxidative stress, are associated with the glycosomes as well. This triggered our interest in their function as glycosomal proteins. Not only the role of the glycosome in trypanosomatid

metabolism is the topic of our research, but also the assembly of the organelle. We are studying the proteins, called peroxins, involved in glycosome biogenesis and particularly the mechanism by which they accomplish the import of matrix proteins.

Enzymes of carbohydrate metabolism

Cloning and characterization of genes for glycolytic enzymes, expression and characterization of recombinant enzymes and inhibitor development.

V. Hannaert, D. Guerra, M.-A. Albert, F. Opperdoes and P. Michels

In previous years, genes of all enzymes of the pathway have been cloned and characterized in our laboratory (3). In addition, we have cloned and sequenced the genes for two isoenzymes of the 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (PFK2/FBPase2), 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 glycolysis in trypanosomatids. Also genes for a number of glycolytic enzymes of *Leishmania* species and *T. cruzi* have been cloned and sequenced; in 2001 the genes for *L. mexicana* phosphoglycerate mutase (PGAM) and *L. donovani* phosphofructokinase (PFK) were characterized (4). The predicted primary structure of almost all trypanosomatid glycolytic enzymes appeared quite different from their mammalian counterparts and some enzymes are even not homologous, rendering several of these enzymes highly promising targets for anti-parasite drugs. Most of the *T. brucei* glycolytic enzymes have been expressed in *Escherichia coli* and purified and their kinetic properties have been determined.

In collaboration with C. López and J.L. Ramirez (Universad Central de Venezuela, Caracas) the gene encoding *L donovani* PFK was cloned and sequenced and its bacterially expressed gene product studied (4). The deduced polypeptide contains a C-terminal type 1 peroxisome-targeting signal (PTS1), -SKV. Like that of the previously characterized *T. brucei* PFK (70 % identity), the sequence showed the highest similarity to inorganic pyrophosphate (PP_i)-dependent PFKs, despite being ATP-dependent. Its kinetic properties were similar to those of the *T. brucei* enzyme. Modeling studies and site-directed mutagenesis were employed to shed light on the structural basis for the unique AMP effector specificity and on ATP/PP_i specificity among PFKs.

The predicted amino-acid sequence of *L. mexicana* PGAM is 74 % identical to that of the *T. brucei* mutase. Both trypanosomatid PGAMs belong to the class of cofactor 2,3-bisphosphoglycerate independent mutases, contrary to the non-homologous cofactor-dependent enzyme of humans. The parasite enzymes have been expressed in *E. coli*, and, upon purification, their kinetic properties have been determined.

During previous years, Prof. W. Hol (University of Washington, Seattle, USA) and Dr. L. Gilmore (University of Edinburgh, UK) and their coworkers have, in collaboration with us, established the crystal structure of several trypanosomatid glycolytic enzymes: aldolase (ALD), triosephosphate isomerase (TIM), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 3-phosphoglycerate kinase (PGK), glycerol-3-phosphate dehydrogenase (GPDH) and pyruvate kinase (PYK). Based on these crystal structures, and on insight in the kinetic properties and catalytic mechanisms, inhibitors of each of these enzymes have been designed and synthesised by our colleagues Prof. J. Périé (Université Paul Sabatier, Toulouse, France) and Prof. M. Gelb (University of Washington, Seattle, USA). 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.

In collaboration with Dr. M. Willson and Prof. J. Périé (Université Paul Sabatier) we also synthesized a number of glucosamine analogues which inhibit both the yeast and the *T. brucei* hexokinase (HXK) (5). The most potent inhibitor of the trypanosome HXK, *m*-bromophenyl glucosamide, did not affect the activity of its yeast counterpart. This compound also effectively inhibited growth of *in vitro* cultured trypanosomes. The structure of the *T. brucei* enzyme, in complex with its *m*-bromophenyl glucosamide inhibitor, was modelled using the crystal structure of the *Schistosoma mansoni* HXK - glucose complex. This model allowed us to explain the mode of action of this inhibitor on the trypanosome HXK.

Also in collaboration with our colleagues in Toulouse, the kinetic mechanism of *T. brucei* PFK has been studied in detail, and its active site has been explored by using a variety of inhibitors derived from the fructose 6-phosphate analogue 2,5-anhydromannitol (6). The best inhibitor was a compound with an electrophilic isothiocyanate

group at position 1; it displayed an irreversible inactivation pattern with a K_i value of 133 μM. The residue involved in the specific inactivation of the parasite enzyme was identified by site-directed mutagenesis, Lys227. Based on this promising result, other compounds are now being developed.

The cloning of the *T. brucei* enolase (ENO) gene was reported last year. The encoded polypeptide has 59 – 62 % identity with the different human enolases.

The kinetic properties of bacterially expressed *T. brucei* ENO are very similar to those of the mammalian enzymes. Furthermore, structure modelling (in collaboration with Dr. D. Rigden, (CENARGEN/EMPRAPA, Brasilia, Brazil) indicated that the overall conformation of the active site of the trypanosomal enzyme is very similar to those of the enzyme from yeast and lobster for which crystal structures are available. However, there are some atypical residues (one Lys and two Cys residues) close to the *T. brucei* active site. These residues could possibly be exploited for the irreversible binding of selective inhibitors. The accessibility of these residues for inhibitors is currently being studied in collaboration with Dr. D. Vertommen (HORM Unit).

The activity of trypanosomatid PYK is allosterically regulated by fructose 2,6-bisphosphate (F-2,6-P_2), contrary to the PYKs from other eukaryotes that are usually stimulated by fructose 1,6-bisphosphate (F-1,6-P_2). The molecular basis of the specificity for the allosteric effector was studied in more detail in collaboration with Drs. D. Rigden (Brasilia) and L. Gilmore (Edinburgh)(7). Based on the comparison of the three-dimensional structure of *Saccharomyces cerevisiae* PYK crystallized with F-1,6-P_2 present at its effector site (R-state) and the *L. mexicana* enzyme crystallized in the T-state, two residues (Lys453 and His480) were proposed to bind the 2-phospho group of the effector. This hypothesis was tested by site-directed mutagenesis. The allosteric activation by F-2,6-P_2 appeared to be entirely abrogated in the mutated enzymes confirming our predictions. In addition, we have prepared two mutants for use as tools to screen the large number of compounds we anticipate from ligand docking, database mining and combinatorial chemistry. Wild-type trypanosomatid PYK has no tryptophan residues, and we have introduced this residue into two different positions near the effector site (F442W and E451W). Both mutants show fluorescence quenching in response to substrates and effectors, and will thus play an important role in screening combinatorial libraries.

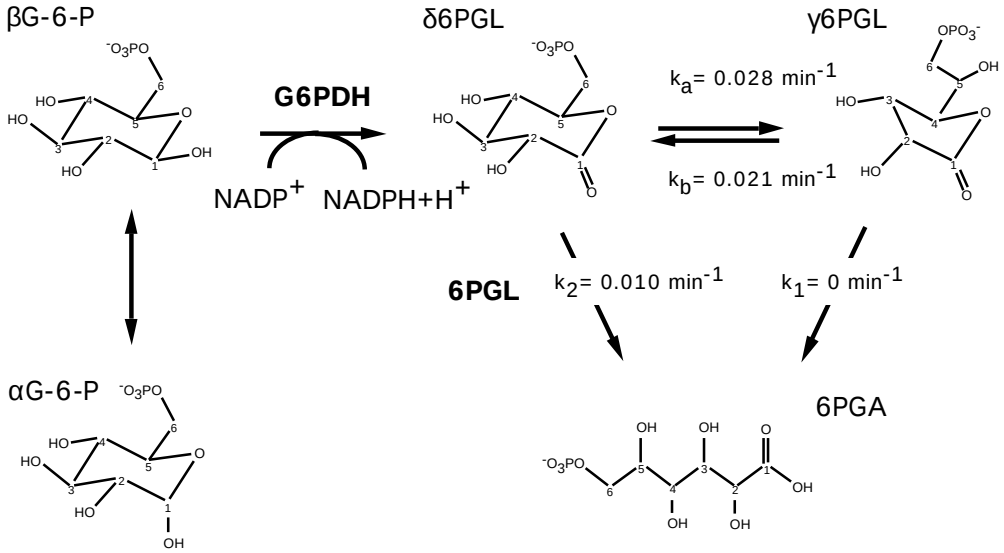

Fig. 1. Proposed scheme for the first two steps of the HMP. Glucose 6-phosphate (G-6-P) oxidation by G6PDH leads to the formation of _-6-phosphogluconolactone (_-6-P-G-L). The two 6-P-G-Ls are in exchange, characterized by k_a, the rate constant of the conversion of _-6-P-G-L into _-6-P-G-L, and k_b, for the reverse reaction. No spontaneous hydrolysis of _-6-P-G-L was measured.

Enzymes of the hexose monophosphate pathway (HPM)

Glucose-6-phosphate dehydrogenase and 6-phosphogluconolactonase

E. Saavedra, P. Michels and F. Opperdoes, in collaboration with F. Duffieux, Université de Paris Sud, France

Previously, we reported the cloning and characterization of *T. brucei* glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconolactonase (6PGL). We have now also cloned, characterized and expressed the corresponding genes of *L. mexicana*.

In collaboration with Dr. Duffieux (Paris) the three-dimensional structure of the *T. brucei* 6PGL is being solved using the technique of nuclear magnetic resonance. So far, the role of this enzyme in metabolism was still questionable, because 6-phosphogluconolactones were believed to undergo rapid spontaneous hydrolysis. By using both ^{13}C and ^{31}P-nuclear magnetic resonance spectroscopy we have characterized the chemical scheme and kinetic features of the oxidative branch of this pathway (Fig. 1) (8). The δ form of the lactone is the only product of glucose 6-phosphate oxidation. It leads to the spontaneous formation of the γ form by intramolecular rearrangement. However, only the δ form undergoes spontaneous hydrolysis, the γ form being a "dead end" of this branch. The δ form is the only substrate for 6PGL. Therefore, the activity of this enzyme accelerates hydrolysis of the δ form, thus preventing its conversion into the γ form. Furthermore, 6PGL guards against the accumulation of δ-6-phosphogluconolactone, which may be toxic through its reaction with endogenous cellular nucleophiles.

The presence of plant traits in the *Trypanosomatidae*

V. Hannaert, J.-P. Szikora, P. Michels and F. Opperdoes, in collaboration with D. Rigden, CENARGEN/EMPRAPA, Brasilia, Brazil

While searching for fructose-1,6-bisphosphatase in trypanosomatid genome databases, we have recently identified in *T. brucei* a complete open-reading frame encoding a homologue of a related enzyme, sedoheptulose-1,7-bisphosphatase (SBPase). The gene has been cloned and sequenced and contains a PTS1, making it a glycosomal protein. SBPase is an enzyme typical of the Calvin cycle of photosynthetic organisms and hence (so far) only encountered in the chloroplasts of green algae and plants. Phylogenetic analysis shows that the closest affiliation of the trypanosome enzyme is with that of the chlorophyte *Chlamydomonas reinhardtii*.

Our recent observation that the glycosomal fructose-bisphosphate aldolase is also closely related to its homologues from plants, which all have a broad substrate specificity and are able to synthesize (and cleave) sedoheptulose 1,7-bisphosphate, suggests to us that these two enzymes must function in tandem in the trypanosomatid HMP. A closer inspection of other genes available in the trypanosome genome database revealed many more sequences with either plant or chloroplast/cyanobacterial affiliation. Most of these enzymes fulfil usually functions in either the Calvin cycle, in glycolysis or in the HMP. We hypothesize that these genes of carbohydrate metabolism probably entered the trypanosomatid ancestor from an algal endosymbiont and that their gene products have later been relocated from the endosymbiont to the host, often to its peroxisomes, after which the remainder of the endosymbiont was lost (9). This may explain the enigmatic presence of glycosomes (i.e. peroxisomes specialized in carbohydrate metabolism) in these organisms.

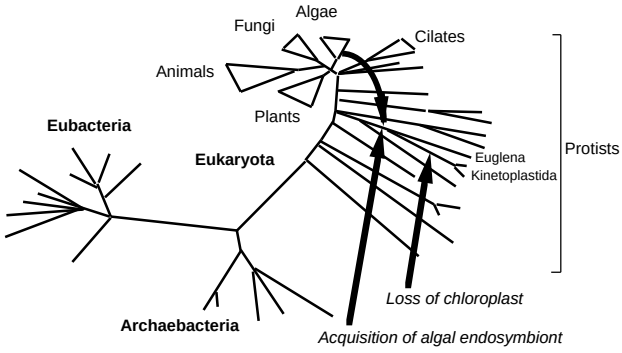

Fig. 2. The "tree of life" based on 16S ribosomal RNA sequences, as modified from Sogin. Indicated are the supposed acquisition of an algal endosymbiont by an organism ancestral to both euglenoids and kinetoplastids. The subsequent loss of this endosymbiont (and its chloroplast) from the kinetoplastid (*Trypanosoma* and *Leishmania*) lineages took place after their separation from the euglenoids, which still have chloroplasts.

Analysis of the control of the glycolytic flux

M.-A. Albert and P. Michels in collaboration with B. Bakker and H. Westerhoff, Vrije Universiteit Amsterdam, The Netherlands, and S. Helfert and C. Clayton, Universität Heidelberg, Germany

Previously, a mathematical model of trypanosome glycolysis was developed based on the kinetic data available for the enzymes involved. This model was able to predict successfully the experimentally determined fluxes and metabolite concentrations in trypanosomes. Our present experiments focus on the experimental determination of the flux control coefficients of the various steps of glycolysis either by their titration with inhibitors, or by the regulation of the expression of the genes coding for the respective glycolytic enzymes. To this end *T. brucei* cell lines have been created in which the expression of HXK, PFK, TIM and glycerol-3-phosphate oxidase (GPO), the mitochondrial enzyme responsible for oxidising the glycolytically produced NADH, can be decreased by RNA interference (RNAi). For that purpose, double-stranded RNA corresponding to the mRNA is synthesised from a transgene under the control of a promoter that is regulated by exogenously added tetracycline. Using these cell lines, it was shown that the growth rate is halved when the TIM level is decreased to 15%; lower TIM levels are lethal. Indeed, simulation by the model predicted that the flux decreases when the enzyme activity drops to about 30%. Upon lowering the level of GPO mRNA, the oxygen consumption was reduced 4-fold and the rate of trypanosome growth was halved. Similarly, reducing HXK expression by RNAi leads to reduction of the glycolytic flux and the growth rate of the cells. Currently, experiments are in progress to establish the quantitative relationship between HXK and PFK expression on the one hand, and the flux and growth rate on the other hand. Similar experiments are being performed with conditional knockout cells which have been created by disruption of the endogenous HXK and PFK genes after the introduction of an newly introduced additional gene copy under the control of an inducible promoter. These experiments will also 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.

Biogenesis of glycosomes

J. Moyersoen, H. Krazy, V. Hannaert and P. Michels

Glycosomal matrix proteins are synthesized in the cytosol and imported post-translationally. The translocation of these matrix proteins across the peroxisomal membrane involves a variety of peroxins. 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. Previously, we reported the cloning and characterization of two cytosolic peroxins, Pex5 and Pex7. These peroxins act as receptors for proteins to be imported into the glycosomal matrix. Pex5 recognizes the PTS1, a signal specified by the three C-terminal amino acids (10), and Pex7 interacts with the PTS2, a nonapeptide motif close to the N-terminus. Both *T. brucei* peroxins, have been expressed as recombinant proteins in *E. coli.* The functional identity of the 70 kDa *T. brucei* Pex5 has been established *in vitro*; the purified protein recognized glycosomal PGK with high affinity.

We have now also cloned and sequenced the *T. brucei* homologues of Pex6, 10, 12 and 14. Their sequences have 32, 21, 25 and 26% identity with the corresponding human peroxins. Pex14 is part of the receptor-docking complex at the glycosomal membrane. Indeed, we could show, by *in-vitro* experiments, its specific interaction with Pex5. The vital importance of Pex14 for bloodstream-form *T. brucei* was demonstrated by RNAi; induced expression of double-stranded Pex14 mRNA resulted in growth arrest of the cells. Pex10 and Pex12 are possibly involved in the translocation process of the receptors Pex5 and Pex7, charged with their ligand, across the organellar membrane, and/or the dissociation of the receptor-cargo complexes at the matrix site of the membrane. These peroxins contain a C-terminal zinc-binding RING domain known to be involved in protein-protein interactions. 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 either involved in membrane fusion processes which lead to the formation of mature organelles or in the receptor recycling from the organellar matrix to the cytosol. So far, we have expressed in *E. coli* the separate ATPase domain of *T. brucei* Pex6. Crystallization trials are currently being performed with this trypanosome Pex6 domain and with various forms of Pex14 and Pex5, and

with combinatons of these latter two peroxins. (J. Choe and Prof. W. Hol).

Analysis of glycosomal membrane solute

Transporters

C. Yernaux and P. Michels

We have started an investigation of glycosomal membrane proteins that might be involved in the transport of glycolytic intermediates or other solutes across the membrane. We have cloned and sequenced two genes coding for putative *T. brucei* glycosomal membrane transporters (TbGAT1 and 2). The amino-acid sequences encoded by these genes are only 30% identical to each other. They are so-called half ABC transporters, containing only a single ATP-binding cassette in their C-terminal half. They are homologous to peroxisomal ABC transporters. Segments of both polypeptides have been expressed in *E. coli* and are being purified; they will be used for antisera production. Investigation of the subcellular localisation and topology of the transporters is in progress. These studies involve transfection of trypanosomes with constructs encoding fusions of segments of the transporters and fluorescent reporter proteins.

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GENETICS OF HUMAN VASCULAR AND SKELETAL DISORDERS AND CEREBRAL TUMORS

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Brendan MCINTYRE, Graduate Student
Emmanuel ROUSSEAU, Graduate Student
Vinciane WOUTERS, Graduate Student
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The basic aim of our research is to get insights into the molecular mechanisms behind human inherited diseases. We are especially interested in disorders affecting the cardiovascular and the skeletal system. In addition, we have initiated studies on 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).

Venous malformations and glomuvenous malformations ("glomangiomas")

P. Brouillard, A. Irrthum, B. McIntyre, V. Aerts, V. Wouters, M. Ghassibé, 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 (GVM, "glomangiomas") are a special subtype of venous anomalies. They are clinically similar to VMs, yet our recent studies have allowed clinical differentiation of these lesions (unpublished). In histologic examination, the pathognomonic sign of GVMs 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* (1). In contrast to inherited VMs, inherited glomuvenous malformations do not link to the TIE2/TEK gene. Instead, they link to *VMGLOM*, a locus on chromosome 1p21 (2). Analysis of the linked families allowed us to observe linkage disequilibrium between certain markers and the phenotype (3). Thus, the locus was narrowed to 1.48 Mbp, covered by a single YAC. Using this single YAC as template, we created a PAC (P1 bacterial artificial chromosome) contig for the reduced VMGLOM region and localized expressed sequences, and thus genes, into the locus. Characterization of these novel positional candidate genes recently led to the identification of the mutated gene, that we named "glomulin" (4). This novel factor does not have sequence identities to known proteins, nor does it contain known functional domains.

Thus, its molecular function is unknown. However, Northern-blot hybridizations showed that glomulin is widely exressed, as all analysed RNAs contained it (4). This is expected if the gene is important for the vasculature, as almost all tissues are invaded by blood vessels. However, we do not currently know, which cell types express glomulin.

As most of the identified mutations cause premature STOP codons in the coding sequence of glomulin (Fig. 1), loss-of-function is the most likely mechanism causing the disorder (4). Furthermore, we hypothesized that as the lesions are localized, a somatic second hit might be needed in the normal allele of the glomulin gene, for lesions to develop. We have obtained proof for this from one lesion (4), but additional lesions need to be studied to confirm these promising results. To study glomulin function, we have cloned about 20kb of the murine glomulin gene (unpublished). This data has been used to create a construct for inactivating the glomulin gene by homologous recombination in murine embryonic stem cells. As the construct contains the lacZ marker gene within the glomulin sequence, homologous recombination would lead to an allele expressing β-galactosidase instead of glomulin. Thus, murine embryos containing such cells could be used to study the expression and the role of glomulin in development and angiogenesis.

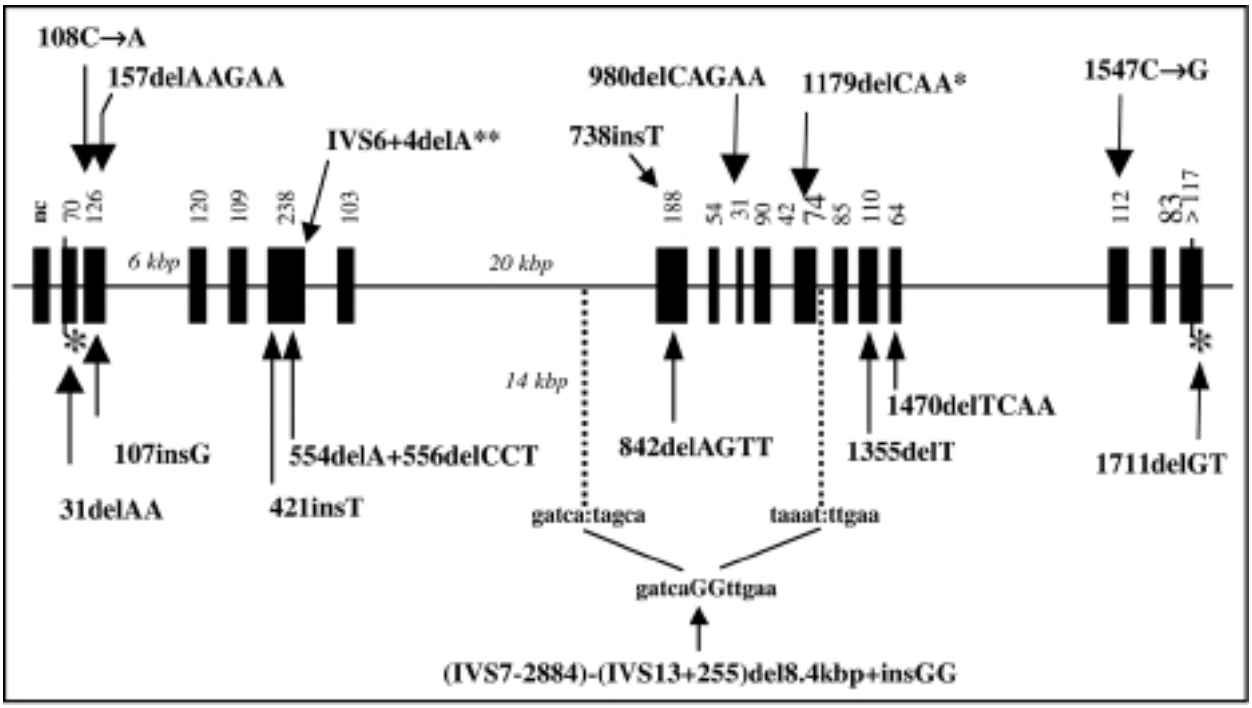

Fig. 1. Glomulin gene – structure and mutations. The size of exons and of the three largest introns are given; other introns are to scale. Exon 1 is noncoding (nc), exon 2 contains the translation start site, and exon 19 contains the TGA stop codon. Above the sequence line, the white arrowheads indicate differences versus FAP48 cDNA (i.e., a new, 85 bp exon and an extra G), the single asterisk (*) indicates a single amino acid deletion, the double asterisk (**) indicate a splite-site mutation, and the box indicates the second-hit mutation, 980delCAGAA; the other three mutations cause immediate stop codons. Below the sequence line, frameshift mutations leading to premature stop codons are indicated, as are sequences of breakpoints of 8.4-kb deletion with GG insertion. The “FAP48” line indicates exons encoding FAP48.

Lymphedema

A. Irrthum, L. Boon and M. Vikkula in collaboration with K. Devriendt, KUL

Primary lymphedema can occur at birth (Meige's disease) or at puberty (Milroy disease). We identified a family in which primary lymphedema was present at birth in several family members. Genetic studies confirmed linkage to 5q33-34 and led to the identification of a mutation in the VEGFR3 gene (5). In vitro expression studies demonstrated that the mutated receptor has lost its autophosphorylation capacity (5). The continued studies have recently led to the identification of linked candidate regions in a family with supposedly recessively inherited congenital lymphedema (unpublished). The identification of the mutated gene should shed further light into factors that are important for lymphatic development

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 Virginia Sybert, Washington University, Seattle, USA

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. These studies, led to the discovery 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 (6). 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 verified the length of the KRIT1 cDNA. Using *in silico* cloning we identified 8 novel exons, four of which are translated (7).

In addition to these studies, we have performed a genome-wide linkage mapping on families with inherited capillary malformations. Large parts of the genome were excluded, finally leading to the identification of a linked locus (8). Identification of the causative gene is an ongoing important project.

Cardiopathies

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

The cardiovascular system may encounter developmental problems affecting the heart. 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 possibly hereditary cardiopathies. In two families, in which atrial septal defect is associated with progressive atrioventricular conduction defect, we identified two novel mutations in the *CSX/Nkx2.5* gene (9) (Fig. 2), an important transcription factor for cardiac development. Identification of mutation carriers is crucial, as in the few studied families the first "symptom" has sometimes been sudden death. Identification of mutations allows genetic testing in the respective families, enabling tight follow-up and preventive pacemaker implantation.

Ongoing studies focus on various forms of atrio-ventricular septal defects and heterotaxia.

Osteoarthrosis and osteochondrodysplasias

M. Ghassibé, V. Wouters, M. Vikkula, in collaboration with D. Manicourt, B. Bayet, R. Vanwijck, N. Revencu and Ch. Verellen-Dumoulin, St-Luc, UCL)

Our main project is in collaboration with Centre labio-palatin, St Luc, to unravel the molecular background of syndromic and non-syndromic cleft lip and/or palate. Numerous blood samples of affected individuals, and their parents and siblings have been collected. This will be continued so as to allow us to do association studies in the future. In addition, collaboration with the cleft lip and palate center of the CHRU Lille has been initiated. These studies have recently led to the identification of mutation causing Van der Woude syndrome (unpublished).

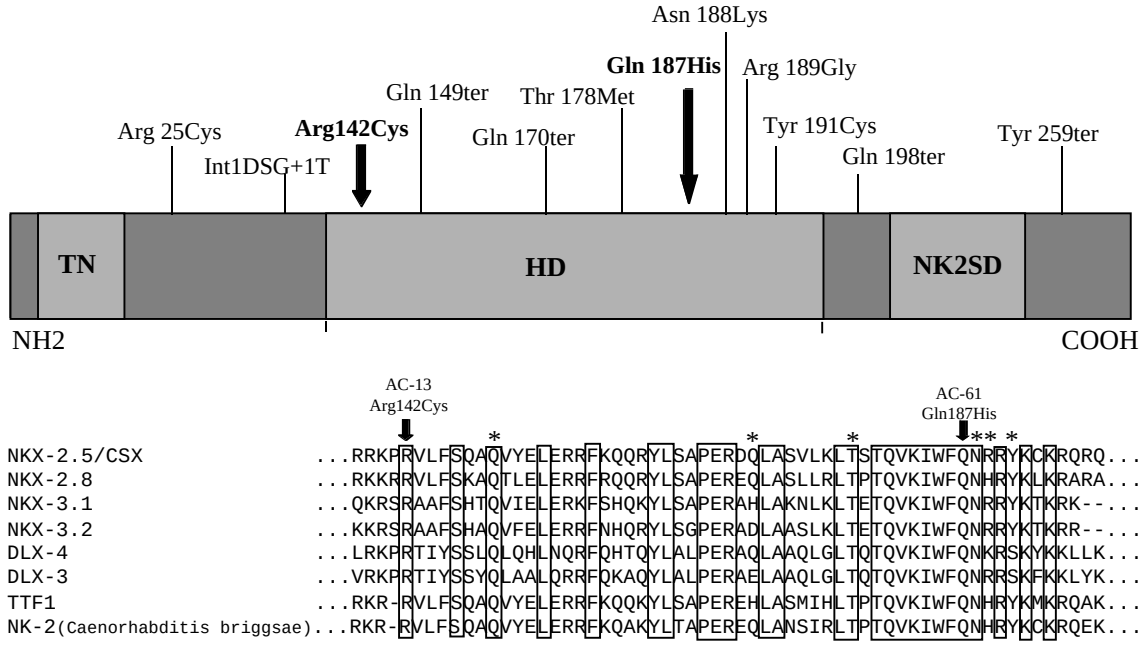

Fig. 2. Schematic representation of CSX/NKX2.5 with tinman domain (TN), homeodomain (HD) and NK2 specific domain (NK2SD). Ten published mutations and two novel missense mutations (bold) from families AC-13 and AC-61 marked above. Top, phenotypes other than ASD with AV block associated with each mutation. For two most 5' mutations, no individual with "ASD with AV-block" reported. Bottom, multiple alignement of amino acid sequences of homeodomains of different human NK-2 proteins and an NK-2 orthologue. *, previously described mutation,. ↓, AC-13 and AC-61 mutations (9). Conserved amino acid boxed.

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 especially interested in two types of cerebral tumors: oligodendrogliomas and ependymal tumours. Using DNA, extracted from formalin-fixed and paraffin-embedded tissues, we have performed loss-of-heterozygosity testing. A restricted screening was performed in a number of oligodendroglial tumours as well as in a large series of ependymal tumours. For oligodendrogliomas, this allowed us to identify and define specific histological characteristics for tumors that have lost chromosome 1p and 19q and that are known to have a preferable response to chemotherapy (10). In addition, we identified methylation difference in ependymomas (unpublished).

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THEILER'S ENCEPHALOMYELITIS VIRUS

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Theiler's murine encephalomyelitis virus (TMEV or Theiler's virus) is a murine picornavirus responsible for infections of the central nervous system. Strains of Theiler's virus 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 reminiscent of those found in human* ***multiple sclerosis****. The genome of Theiler's virus is an 8 kb-long positive strand RNA molecule (Fig. 1).*

Theiler's virus is 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 can be manipulated 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, thus evading the immune response. We analyze viral and cellular determinants of tropism and persistence. We notably analyze the interferon α/β response and the inhibition of this response by Theiler's virus, in the context of the central nervous system.

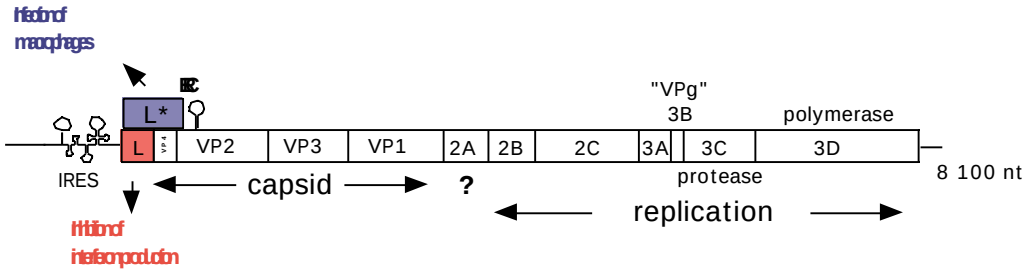

Fig. 1. Genome of Theiler's virus. A large open reading frame encodes a 2000 amino acid-long polyprotein that is cleaved, by autoproteolytic activity, into 12 mature proteins. An additional protein (L*) is encoded by an alternative open reading frame, overlapping regions L, VP4 and VP2. Translation of both ORFs is driven by an Internal Ribosome Entry Site (IRES) present in the 5' non-coding region of the genome (1). Protein L* was shown to facilitate the infection of macrophages and viral persistence (2, 3). Protein L inhibits immediate-early type-I interferon (4). The role of protein 2A is unknown (5). 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 replication signal has been discovered in the VP2 coding sequence and is denoted CRE for "cis-acting replication element" (6).

Inhibition of type-I interferon production by the leader protein

V. van Pesch, S. Delhaye, and T. Michiels

The leader (L) protein encoded by Theiler's virus is a 76 amino acid-long peptide containing a zinc-binding motif. We showed recently that the L protein inhibits the production of type-I interferons (IFNs) (4). Inhibition turned out to be specific for α4 and β interferons, interferon subtypes that are known to be activated early in response to viral infection, therefore referred to as "immediate-early interferons".

A potential target of the leader protein is IRF-3, as this cell factor is known to activate specifically the transcription of IFN-β and IFN-α4 genes. 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.

Production of immediate-early interferons (α 4 and β) is generally considered to be required for transcriptional activation of the other IFN-α subtype genes. However, total IFN-α transcription appeared to be induced rather than repressed in wild-type virus-infected L929 cells, in spite of the inhibition of the α 4 and β IFNs. The IFN-alpha subtypes expressed in the absence of these early interferons are currently under identification.

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. 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.

In contrast, interferon receptor-deficient mice were readily infected by both the wild-type and the mutant viruses. These data confirm the crucial role played by the interferon response against Theiler's virus infection and the anti-IFN role of the L protein. However, IFN production inhibition was not complete in vivo. Modulation rather than blockade of the IFN response might be viewed as a viral strategy toward long-term persistence in the host.

Influence of the L* protein on macrophage infection and viral persistence

O. van Eyll, B. Michel, 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 (2, 3, 7).

We compared the phenotype of L* mutant viruses carrying either an AUG to ACG mutation of the initiation codon or a stop codon mutation introduced in the L* ORF. Surprisingly, viruses carrying a stop codon mutation (and thus expressing a truncated L* protein) had a dramatically impaired ability to persist in the central nervous system of the mouse while mutants bearing the AUG to ACG mutation persisted almost as well as the wild-type virus. Our data suggest that L* could be expressed from an ACG initiation codon. This would be the first example of picornavirus IRES-driven non-AUG translation initiation. In addition, it indicates that neurovirulent strains can also produce the L* protein.

Current efforts aim at characterizing the function of the L* protein in the infection of macrophages. Macrophages are indeed key players in the demyelinating disease induced by Theiler's virus, being simultaneously effectors of the immune response and targets of viral infection.

Interaction of Theiler's virus with the cell

K. Jnaoui, and T. Michiels

Despite many efforts, the cellular receptor for Theiler's virus has not yet been identified. We analyzed the interaction between the virus and the cell.

On the one hand, we obtained and analyzed a series of virus variants adapted to grow on various cell lines (8-10). 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. The fact that optimal infection of a specific cell lines requires adaptation of the viral capsid suggest that the receptor or co-receptor for the virus is polymorphic.

On the other hand, we isolated mutant cell lines that became resistant to the entry of the GDVII strain of Theiler's virus, but which retained susceptibility to persistent strains (5). This shows that infection of a given cell lines by different strains of Theiler's virus involves at least partly distinct factors.

Persistent strains but not neurovirulent strains of Theiler's virus were reported to bind sialic acid. This interaction involves 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 neurovirulent GDVII strain could interact weakly with 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 (9).

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.

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ANTIVIRAL IMMUNITY AND PATHOGENESIS

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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 frequent mutations or latency, or even to impair this system, which often leads to diseases such as autoimmunity or immune deficiencies. Our project is to investigate, in murine models, some aspects of these relations between viruses and the immune system.

Viral infections result in a dramatic increase in the proportion of IgG2a

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 activate the complement system, in contrast to IgG2a, another major isotype 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. A dual regulation of antibody responses by gamma-interferon and interleukin-6 explains this isotypic bias (1, 2). 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. Using a model of infection with lactate dehydrogenase-elevating virus (LDV), we could demonstrate that IgG2a antiviral antibodies are indeed more efficient than other isotypes to protect mice against a fatal polioencephalomyelitis induced by the virus (3, 4). 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 (5, 6). Indeed, different subpopulations of those cells, called Th1 and Th2, respectively, are distinguished in particular by their capability of producing selectively interferon-γ (IFN-γ) or interleukin-4, which can selectively trigger B lymphocytes to produce IgG2a or IgG1, respectively.

Activation of natural killer cells

Many of the influences that viruses may have on diverse immune responses can be explained by the production of pro-inflammatory cytokines (7), including IFN-γ. Therefore, our analysis of the relationship between viruses and the immune system has focused on the activation, by LDV, of cells from the innate immune system that are able to secrete this cytokine, namely the natural killer (NK) cells.

Within a few days after infection, a strong

and transient NK cell activation, characterized by accumulation of this cell population in the spleen, by enhanced IFN-γ message expression and production, as well as by cytolysis of target cell lines was observed. Because NK cells and IFN-γ may participate in the defense against viral infection, we analyzed their possible role in the control of LDV titers, with a new agglutination assay. Our results indicate that neither the cytolytic activity of NK cells nor the IFN-γ secretion affects the early and rapid viral replication that follows LDV inoculation (8).

Activation of macrophages

Activation of cells of the innate immune system includes also macrophages and leads to an enhanced phagocytic activity, with potential detrimental consequences for ongoing autoimmune diseases (9). We have thus analysed whether it was possible to modulate such an activation by treating infected mice with clodronate-containing liposomes. Administration of anti-erythrocyte monoclonal autoantibody to mice resulted in the development of a transient hemolytic anemia. Infection with LDV simultaneously with autoantibody injection was followed by a dramatic enhancement of the anemia, leading to the death of most animals. This viral infection induced an increase in the ability of macrophages to phagocytose *in vitro* autoantibody-coated red cells, and an enhancement of erythrophagocytosis in the liver. Treatment with total immunoglobulin G (IVIg) attenuated the autoantibody-induced disease in uninfected mice, but not in LDV-infected animals (10). In contrast, administration of clodronate-containing liposomes resulted in a delay and a decrease of anemia in LDV-infected mice. This treatment decreased also the *in vitro* phagocytosis of autoantibody-coated red cells by macrophages from LDV-infected animals. Thus, regulation of macrophage activation results in modulation of autoantibody-mediated anaemia and may be considered as a possible treatment for autoimmune diseases that involve phagocytosis as a pathogenic mechanism.

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HUMAN TUMOR IMMUNOLOGY*

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In association with the Ludwig Institute :see ***Analysis of T cell responses of vaccinated cancer patients*** (research at LICR, Brussels)

Human tumor cells bear antigens that are not present on normal cells and that can be specifically recognized by autologous CD4 or CD8 T lymphocytes. We contributed to the identification of several tumor-specific antigens, present on melanoma or lung carcinoma cells (1-4). Tumor-specific antigens, such as those encoded by the MAGE genes, have been used to vaccinate melanoma patients with detectable disease. About 20 % of the vaccinated patients display a tumor regression, a frequency that appears well above the level reported for spontaneous melanoma regressions. Nevertheless, the treatment fails in most patients, and this can probably only be improved if a better understanding of the immune responses of the patients is acquired. Our group focuses on such analyses.

A new method for the detection of low level tumor-specific T cell responses

V. Karanikas and T. Aerts in collaboration with D. Colau, Brussels branch of the Ludwig Institute for Cancer Research

The failure of 80 % of the vaccinated patients to show tumor regression could be due to two major causes which are not mutually exclusive : a failure of the vaccine to induce an adequate T cell response or a resistance of the tumor to immune attack. To the extent that the limiting factor for success would be the level of the T cell response to the vaccine, one ought to find a correlation between the occurrences of T cell responses and those of tumoral regression. We focused our efforts on the detection of cytolytic T lymphocytes (CTL) recognizing a peptide encoded by gene *MAGE-3* and presented by HLA-A1. The group of Pierre van der Bruggen showed that the frequency of anti-MAGE-3.A1 CTL in the blood of an individual without cancer is about 4.10^{-7} of the CD8 cells. To detect safely an increase of at least 10-fold of this naive frequency of anti-MAGE-3.A1 T cells, we resorted to an approach based on an *in vitro* restimulation of blood lymphocytes with the antigenic peptide and T cell growth factors over two weeks, followed by labeling with A1/MAGE-3 "tetramers" : fluorescent soluble complexes of HLA-A1 molecules presenting the MAGE-3.A1 peptide. In order to evaluate precursor frequencies, these cultures are carried out in limiting dilution condition. Cells that are labeled with the tetramer are cloned, and their diversity analyzed by TCR sequencing.

This MLPC/tetramer/cloning approach was applied to a metastatic melanoma patient who responded clinically to vaccination with peptide MAGE-3.A1 administered without adjuvant (5). We found that the vaccination induced at least a 100-fold amplification of the anti-MAGE-3.A1 CTL present in the blood, with a postvaccination frequency of 5.10^{-5} of the CD8 cells. Surprisingly, TCR analysis of anti-MAGE-3.A1 CTL clones derived from positive cultures indicated that this CTL response was monoclonal. This finding enabled us to analyze directly the frequencies of blood T cells expressing this TCR, using "clonotypic" PCR amplifications specific for

its Vα or Vβ rearrangement. The frequencies of anti-MAGE-3.A1 CTL measured by this genetic approach closely matched those found by the MLPC/tetramer method. These results demonstrate that some patients vaccinated with a MAGE antigen mount CTL responses, even after vaccination with an antigenic peptide without adjuvant.

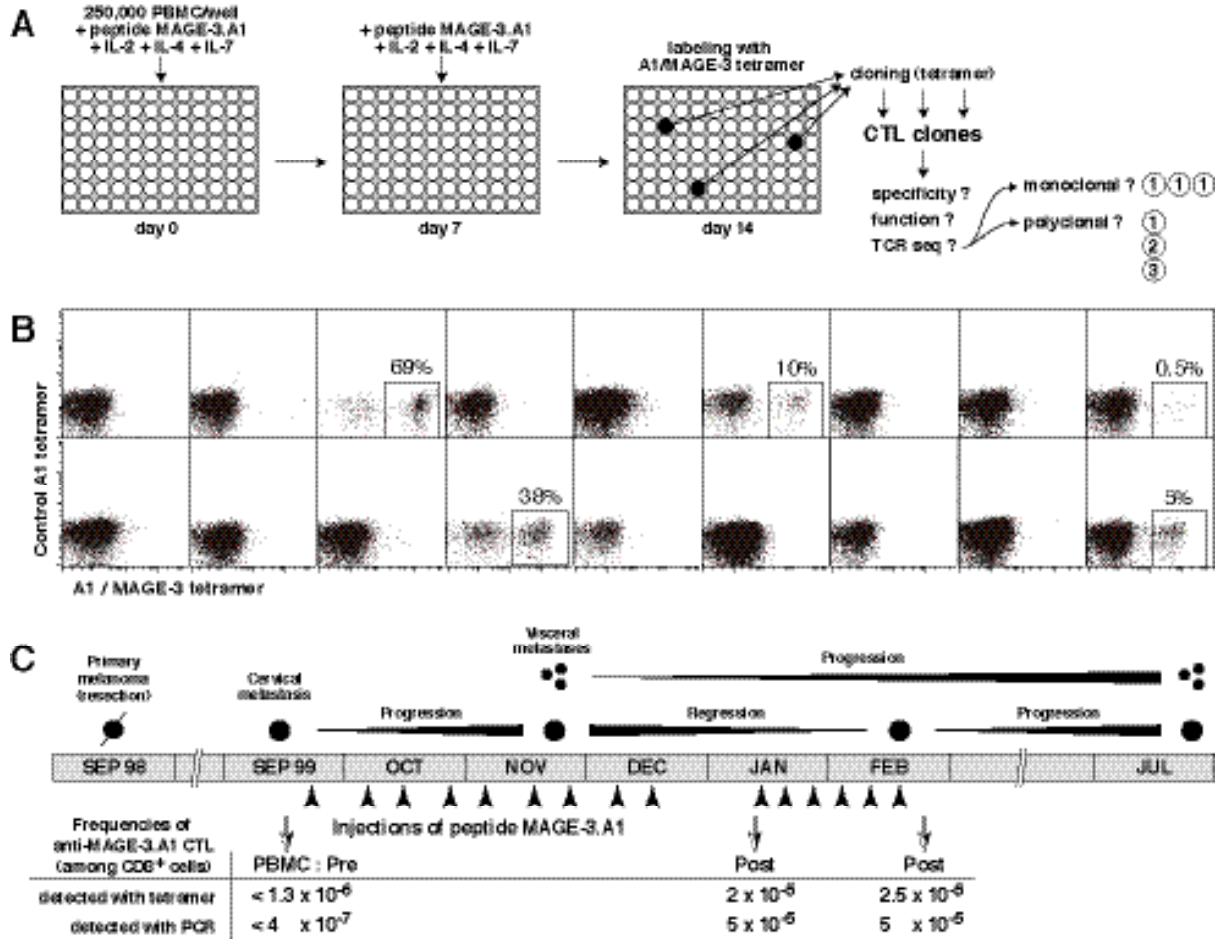

Fig. 1. A. Overview of the MLPC/tetramer/cloning procedure for the analysis of anti-MAGE-3.A1 T cells. **B. Typical results of labeling with A1/MAGE-3 tetramer after two weeks of stimulation**. Groups of 160,000 postvaccination PBMC from patient CP64 were stimulated as described in A. The lymphocytes were labeled on day 15 with tetramer and anti-CD3 and anti-CD8 antibodies. Only $CD3^+CD8^+$ lymphocytes are included in the plots. Two types of HLA-A1 tetramer were used, containing peptide MAGE-3.A1 or a control influenza nucleoprotein peptide. Clusters of lymphocytes specifically labeled with the A1/MAGE-3 tetramer are boxed, and their proportion among the $CD3^+CD8^+$ cells is indicated. **C. Clinical evolution of patient CP64, and frequencies of blood anti-MAGE-3.A1 T cells**.

Anti-MAGE CTL responses in vaccinated patients : Correlation with clinical responses ?

V. Karanikas, T. Connerotte, V. Corbière, C. Muller, C. Mondovits, T. Hanagiri in collaboration with C. Lurquin and D. Colau, Brussels branch of the Ludwig Institute for Cancer Research

We have analyzed the T cell responses of metastatic melanoma patients with detectable disease, following vaccination with a recombinant poxvirus of the ALVAC type, which bears a short *MAGE-3* gene sequence coding the MAGE-3.A1 antigenic peptide. Three out of five patients who showed tumor regression had an anti-MAGE-3.A1 CTL response. For two patients, there was a clear increase in the frequency of anti-MAGE-3.A1 CTL after vaccination : from $< 3.10^{-7}$ to 3.10^{-6}

in one patient and from 8.10^{-7} to 3.10^{-3} in the other. These two CTL responses were monoclonal. In the third patient the anti-MAGE-3.A1 CTL frequency did not increase after vaccination, but the TCR analysis indicated that one CTL clone had been amplified. These results indicate that, like peptide immunization, ALVAC immunization usually produces a monoclonal response. Only two anti-MAGE CTL responses were observed among 12 patients who did not show tumor regression following ALVAC vaccination. These results suggest that there is a certain correlation between CTL response and tumor regression, but this will need confirmation with larger numbers (6, 7).

Analysis of regressing tumors

C. Germeau, E. Henry, C. Muller in collaboration with W. Ma, C. Lurquin, B. Lethé, Brussels branch of the Ludwig Institute for Cancer Research

If clinical responses correlate with immunological responses, anti-vaccine T cells probably initiate tumor rejection. One possibility is that they reach and destroy the tumor. This could be examined with a clonotypic PCR applied to a tumor specimen biopsied at the time of regression. Preliminary results obtained from two patients suggest that the anti-vaccine CTL clones found in the blood were not enriched in tumors.

Another possibility is the involvement of T cells recognizing tumor-specific antigens absent from the vaccine. Detection of such CTL requires the establishment of an autologous tumor cell line, and stimulation of blood lymphocytes with the tumor cells to derive tumor-specific CTL clones. The specificity and the TCR diversity of these CTL clones can then be analyzed. We have started this analysis with a melanoma patient who displayed tumor regression after vaccination with the ALVAC/MAGE construct. We measured the blood frequencies of CTL recognizing the vaccine antigen, MAGE-3.A1, and of CTL recognizing other, undefined, tumor antigens. We observed that the anti-vaccine CTL frequency rose from $< 5.10^{-7}$ to 3.10^{-6} of the CD8 after vaccination. On the other hand, the anti-tumor CTL frequencies were already about 10^{-3} of the CD8 before vaccination, and increased sligthly after vaccination. TCR analysis of panels of anti-tumor CTL clones indicated that one clone had been amplified after vaccination. We identified the antigen recognized by this anti-tumor CTL : it is a peptide presented by HLA-A2 and encoded by gene *MAGE-C2*, which is specifically expressed in tumors. These results suggest that other tumor-specific CTL than those that recognize the vaccine antigen can be stimulated after vaccination.

We then used TCR-specific PCR to detect the anti-vaccine (MAGE-3.A1) and the anti-MAGE-C2.A2 CTL in a tumor sample collected from this patient after vaccination. The results are very clear : the anti-vaccine CTL is present in the tumor at the same frequency than in the blood, whereas the anti-MAGE-C2.A2 CTL is considerably enriched (at least 100-fold) in the tumor.

These results suggest that tumor-specific CTL others than those that recognize the vaccine antigen may participate in the tumor rejection that follows vaccination. A likely scenario involves « antigen spreading » : an initial and low level CTL response against the vaccine peptide leads to the destruction of a small number of tumor cells, followed by the processing and presentation of other tumor antigens. New waves of CTL may then follow, that recognize these antigens. This would explain how tumors can be rejected with a very low frequency of anti-vaccine CTL. We are now trying to validate this model with other patients. We will also examine the functional properties of the anti-vaccine T cells. It is indeed surprising that a tumor rejection takes place after the activation of a few anti-vaccine lymphocytes, even though many other tumor-specific T cells were already present before vaccination. By comparing these two types of lymphocytes, we may understand why only the anti-vaccine lymphocytes appear to initiate tumor rejection.

A new lung tumor antigen

P.G. Coulie in collaboration with J.-F. Baurain, Department of Oncology, Cliniques Universitaires St Luc, Université de Louvain, Brussels, and H. Echchakir, Laboratoire Cytokines et Immunologie des tumeurs Humaines, Institut National de la Santé et de la Recherche Médicale, Institut Gustave Roussy, Villejuif, France)

We have identified an antigen recognized on a human large cell carcinoma by an autologous tumor-specific CTL clone that was derived from mononuclear cells infiltrating the primary tumor (8). 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. Although he did not receive chemotherapy or radiotherapy, the patient 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.

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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 1997 the ICP has been able to allocate the following fellowships, entirely supported by our donors :

the "Haas-Teichen" fellowship to Christian SINGER
the "Hubert and Aldegonde Brenninkmeijer" fellowship to Aoife BOYD
the "Michel de Visscher" fellowship to Norton HEISE
the "Petrofina" fellowship to Sandrine MARIE
the "Philippe Delori" fellowship to Janine BILSBOROUGH
the "Société Générale de Belgique" fellowship to Mahfuzur SARKER (till May 1997) and
to Ivica KRAL'OVA (from May 1997)
the "Van Eessel" fellowship to Sylvie MARCHE

An "Art & Science" Gala Dinner was held on October 20, 1997 at the Museum of Ixelles to celebrate the new name of the Institute. It was attended by 240 representatives of the academic and economic worlds. This philantropic event yielded a substantial revenue.

Our gratitude goes to all the generous donors.

Ludwig Institute for Cancer Research (LICR),

Brussels branch

The Ludwig Institute for Cancer Research

Cancer is a major concern in human health. The prospects for bringing cancer under control require linked innovative basic and clinical research. In this view, Daniel K. Ludwig created in 1971 the Ludwig Institute for Cancer Research, an international organization bringing together scientists and clinicians from around the world. Ludwig investigators are recognized leaders in many areas of science, involving genetics, bioinformatics, immunology, virology, cell biology and signal transduction.

Thierry Boon

Faithful to the organizing principles laid down by Mr Ludwig, the Institute conducts its research through ten Branches, located in seven countries. The Branch structure allows the Institute to interact with a number of different research and clinical environments. Each Branch is focused on a research program defined by the Branch Director in relation with the overall objectives of the Institute. The Branches are established in association with University Hospitals, to stimulate close collaborations between research laboratories and the clinic. By organizing and controlling its own clinical trials programs, the Institute has indeed created a continuum that integrates laboratory and clinical research.

Branch staffs vary in size from 30 to over 70, and internationally the Institute employs some 600 scientists, clinicians and support personnel. The quality of the research is monitored on an ongoing basis by the Institute's Scientific Committee and by an external peer review process.

The biological properties of any given cancer cell constantly change, allowing tumors to spread and become more aggressive. To overcome these obstacles, the Ludwig Institute has developed a broad-based discovery program that seeks to understand the full complexity of cancer. Research is organized according to the four major programmatic themes that define the Institute : genetics, cell biology, cell signalling and immunology.

I. TUMOR IMMUNOLOGY AND IMMUNOTHERAPY

OVERVIEW

T lymphocytes are capable of destroying cells that harbour viruses because they recognize viral antigens on their surface. They do so with great efficacy and specificity and, together with the anti-viral antibody response, they usually provide full recovery from viral diseases. For cancer the situation is very different : clearly the immune system does not eliminate most overt cancers. But cancer cells have long be known to differ from normal cells in many respects and it has been a long-standing hope of immunologists that cancer cells express abnormal antigens which could be recognized by the immune system. The existence of tumor-specific antigens was considered to have two major implications : the existence of cancer immunosurveillance and the feasibility of cancer immunotherapy. The hypothesis of cancer immunosurveillance implies that most cancers are eliminated at a very early stage by an immune rejection process. One could add that the immune system, while failing to eliminate some tumors, may nevertheless slow them down at all stages of their progression. The existence of immune surveillance has long been controversial but it is supported by recent evidence obtained with mouse tumors. Cancer immunotherapy is based on the notion that it is possible to artificially improve the response to tumor antigens to make it reach its full potential. Unlike responses directed against viral antigens, anti-tumoral responses may not have been perfected throughout evolution, because escaping cancer probably conferred little or no selective advantage.

Our interest in tumor immunology started with a fortuitous observation made with a mouse tumor which was strictly non-immunogenic. Mice from which this tumor was removed by surgery did not show any protection against a challenge with the same tumor cells. We observed that by treating the tumor cells in vitro with a mutagen we obtained tumor cell mutants that were rejected in the mice by a T lymphocyte mediated process (1). Remarkably the mice that had rejected these "tum-" mutants showed a degree of protection against a challenge with the original non-immunogenic tumor cells (2). The pattern of protection indicated that the tum- mutants owned their phenotype to the acquisition of new strong tumor rejection antigens. The response against these antigens evidently created conditions that facilitated a response against weaker antigens present on the original tumor, a phenomenon which has recently been named "epitope-spreading". These observations were found to apply to all mouse tumors, including spontaneous tumors (3). This led to two conclusions. First, all mouse tumors bear tumor-specific antigens recognized by T cells even though many of them are non-immunogenic.

Second, it is possible to create conditions that favor the T lymphocyte responses against the tumor antigens.

On the basis of these findings we launched an effort to identify the antigens recognized on mouse tumors by T cells. We focused our effort on mouse tumor P815. A first step was to obtain in vitro cytolytic T lymphocytes (CTL) that specifically lysed the P815 cells (4, 5). Then, with considerable help from Jean-Charles Cerrotini and other members of the Lausanne branch, we obtained stable CTL clones directed against tumor antigens of P815. Antigen-loss mutants of P815 were obtained in vitro by selection for resistance to lysis by a CTL clone (6). In addition, we observed that antigen-loss is a mechanism which is used by P815 tumors to escape T cell responses in vivo. The next step was the production of genomic libraries from antigen-bearing cells and the transfection of the DNA into antigen-loss variants. Antigenic transfectants could be detected on the basis of their ability to stimulate the proliferation of the relevant CTL clone and the genes coding for the antigens could be retrieved from these transfectants by using appropriate cosmid technology (7).

About at that time, Alan Townsend showed that influenza virus antigens derived from proteins that remained inside the cells could be detected by T cells. These antigens are recognized by T cells as small peptides presented by class I major histocompatibility complex (MHC) molecules. Our contribution was to show that this also applied to the proteins encoded by the genome of the cell (7, 8). Thus there is a permanent T cell based immune surveillance of the cellular genome and genetic abnormalities can result in the presentation of new antigens leading to elimination by T cells. Our results also demonstrated that there are two major genetic processes that produce tumor-specific antigens. The first is the acquisition of mutations by the cancer cell, which generate peptides which, because of an amino-acid change, either become capable of binding to MHC molecules or contain a new epitope (8). The second is the expression by the tumor of a gene which is not expressed in the normal cells of the adult (9)(Fig. 1).

Around 1985 we began to examine whether the results obtained in mice could be extended to man. We focused our efforts on melanoma because, contrary to most tumors , samples of metastatic melanoma can be converted into stable cell lines fairly frequently. Stimulation of T lymphocytes with autologous melanoma cells produced cytolytic T cells that appeared to lyse the tumor cells specifically. Stable CTL clones were obtained and immunoselection of antigen-loss variants indicated that, like mouse tumors, human tumors express not one but several antigens that are recognized by autologous CTL (10, 11). A cosmid genomic library derived from a tumor cell was then transfected into an antigen-loss variant and this led to the identification of the first gene coding for a human tumor-specific antigen recognized by T cells (12). This previously unknown gene was named *Mage* and it was soon found to be expressed in many melanomas and not in normal cells.

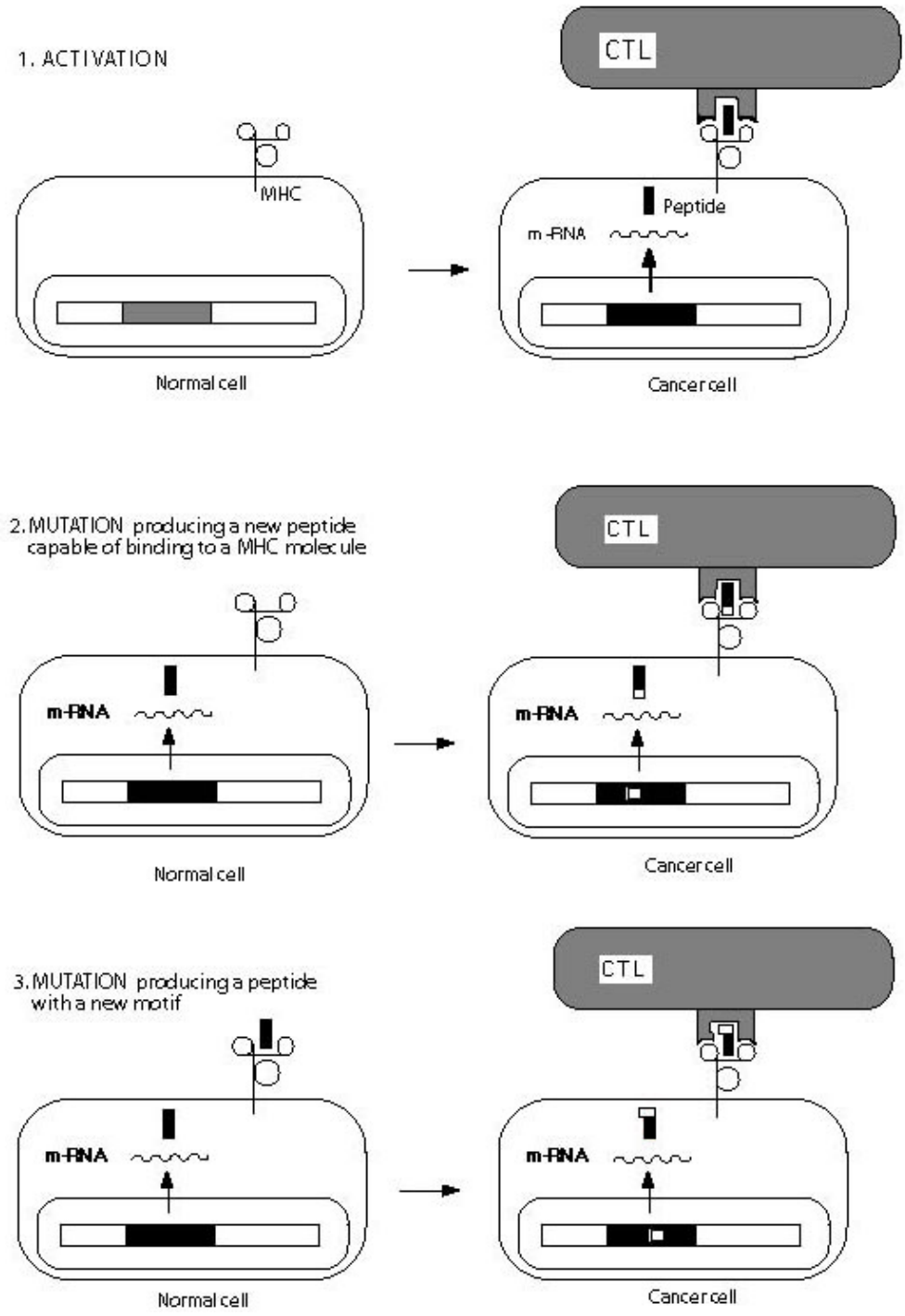

Fig.1

Gene *Mage* belongs to the *Mage-A* gene cluster, which comprises 12 genes (13). It is located on the X chromosome. The homologous families *Mage-B* and *C* are located on the same chromosome. All these genes have the same expression pattern. They are expressed in many tumors of various histological types and not in normal cells with one exception, male germline cells (13). It is therefore

appropriate to refer to these genes by the term cancer-germline genes. It is important to note that the germline cells do not express HLA molecules and therefore cannot present antigens to T cells. The antigens encoded by *Mage* genes appear therefore to be strictly tumor-specific as far as T cell responses are concerned, contrary to what is suggested by the term cancer-testis antigen which is often used to refer to these antigens. The activation of *Mage* genes in cancer and germline cells is not due to the presence of specific transcription factors. It is caused by the demethylation of the promoter in these cells (14). In cancer cells this appears to be a consequence of a global demethylation, which is observed in many cancer cells.

Using similar approaches we identified other families of cancer-germline genes, such as the *Bage* and *Gage* families (15, 16). cDNA enrichment approaches capitalizing on the cancer-specific pattern of expression was used to find additional cancer-germline gene families such as *Lage*, *Sage* and *Hage*. Another approach, Serex, based on antibody responses of cancer patients was initiated by Michael Pfreundschuh. It enabled him to identify the SSX and SCP-1 families. Using this approach, members of the New York Branch identified a second member of the Lage family, named NY-ESO-1, shortly after our discovery of *Lage-1* (17). It is likely that by now most of the cancer-germline genes have been identified.

Antigens encoded by cancer-germline genes ought to be very suitable as therapeutic vaccines for cancer-patients as they are strictly tumor-specific and present on a large proportion of tumors. But genes that are strongly overexpressed in cancer cells relative to normal cells could also be a source of acceptable antigens for cancer immunotherapy. An example is gene *Prame*, which is expressed at a high level in a very large proportion of melanoma and other tumors (18).

Gene mutation was also found to be a major source of human tumor-specific antigens. Interestingly, several mutations that were found to be antigenic also appear to play a role in oncogenesis. One interesting example is a mutation in cyclin-dependent kinase-4 ,which prevents the binding of this protein to P16 (19). As a result CDK4 permanently phosphorylates Rb causing excessive cell cycling. Another example is a mutation in caspase 8, which reduces the sensitivity of cells to pro-apoptotic factors (20). Unfortunately, when a tumor-specific antigen results from a mutation it is expressed on an extremely small proportion of tumors. This precludes the use of this class of tumor-specific antigens as cancer vaccines.

Finally, we observed that CTL of melanoma patients can respond to antigens encoded by melanocytic differentiation genes, such as tyrosinase and Melan-A (also referred to as Mart-1)(21, 22). This is surprising as one would have expected natural tolerance to put a thight block on responses against such self antigens. Other groups proceeded to vaccinate melanoma patients with these antigens and observed tumor regressions in some patients.

The peptides that are presented by MHC molecules at the cell surface result from the degradation of intracellular proteins by the proteasome in the cytosol. The peptides are then transported into the endoplasmic reticulum where they combine with newly synthesized MHC molecules on their way to

the cell surface. The proteasome plays a central role in this pathway, known as the class I antigen processing pathway. Some cells, such as dendritic cells and cells exposed to interferon-gamma, express a different type of proteasome named immunoproteasome, whose catalytic activity is slightly different from that of the standard proteasome. We have shown that a number of human tumor antigens are not produced with the same efficiency by the two proteasome types, some of them are produced only by the standard proteasome whereas others are produced exclusively by the immunoproteasome (23, 24). This means that the peptide repertoire displayed at the cell surface depends not only on the proteins expressed by the cells but also on the type of proteasome they harbor. This parameter should be considered in the context of immunotherapy.

For the vaccination of cancer-patients with a tumor antigen recognized by T lymphocytes, several forms of the antigen can be used. These include antigenic peptides, whole protein, recombinant defective viruses carrying a sequence coding for the antigen, or naked DNA. In addition, dendritic cells derived from blood cells of the patient can be reinfused into the patient after being pulsed with antigenic peptides or protein or after being transfected with encoding cDNA or RNA. The simplest vaccines are the antigenic nona- or decapeptides. However each peptide binds and can be presented only by the protein encoded by one or a few HLA alleles. It is therefore necessary to identify many antigenic peptides to be able to provide an adequate vaccine to the majority of cancer patients. The identification of these antigenic peptides on the basis of the gene sequence involves the obtention of a T cell clone that recognizes this antigen. We have devised approaches to obtain such CD8 T cell clones by stimulating T cells with dendritic cells infected with a recombinant virus carrying the encoding gene (25). CD4 T cell clones have been obtained by stimulation with dendritic cells pulsed with the relevant protein. As a result we have now identified a large number of antigenic peptides encoded by genes *Mage-1* and *Mage-3* (Fig.2).

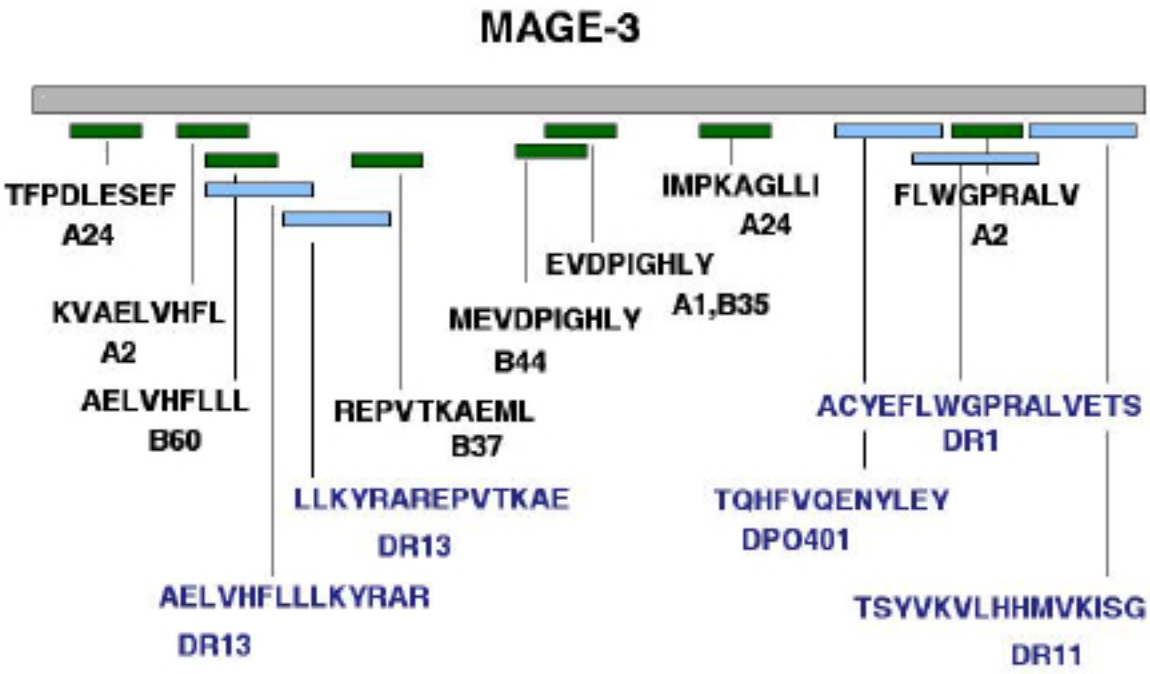

Fig.2

Our first clinical trial involved the vaccination of metastatic melanoma patients with an antigenic peptide which is encoded by *Mage-3* and presented by HLA-A1. Seven patients out of 26 showed evidence of tumor regression (26, 27)(Fig.3). No toxicity was observed. We then examined in a series of small trials whether changes in the vaccine modalities would significantly improve the outcome. We tried more frequent injections of the Mage-3.A1 peptide, combination with an adjuvant or combination with another Mage peptide binding to either a class I or class II HLA molecule. No improvement was observed. Similar results were observed after vaccination with the Mage-3 protein or with an ALVAC recombinant virus coding for *Mage* sequences. In these trials we failed to detect CTL responses against the vaccine. Even though our approach lacked sensitivity, this indicated that the CTL responses were weak at best. To sum up the observations made on about 200 patients, we can say that some evidence of tumor regression is observed on about 20% of the patients with half of them, i.e. 10%, showing complete or partial clinical responses.

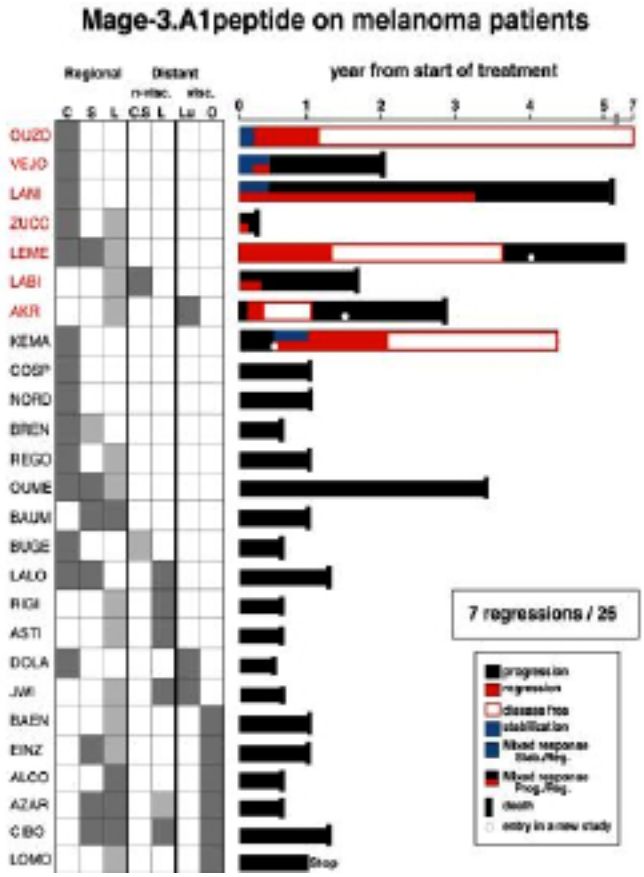

Fig.3

To explain why our vaccinations fail to exert any detectable effect on the tumors of 80% of the patients one can consider two, unfortunately non-exclusive, causes. First, the anti-vaccine CTL response might be inadequate. Second, the tumor might be resistant to immune attack. We set out to analyze the first possibility, considering that if the level of the CTL response to the vaccine was a limiting factor for clinical efficacy, we ought to observe a correlation between the observations of CTL responses ant those of tumor regressions. We therefore developed highly sensitive approaches for the detection of CTL responses combined with an analysis of the T cell receptor diversity of the responding CTL (28). These approaches are beginning to show a correlation between CTL responses and tumor regressions. If this trend is confirmed, it will be crucially important to understand why some patients make CTL responses to the vaccines whereas others do not. When patients vaccinated with antigenic peptides or vaccinia-like recombinant viruses produce a T cell response, it is monoclonal (28). In contrast, patients vaccinated with peptide-pulsed dendritic cells produce either polyclonal responses or no response. We will engage in a systematic study of patients recruited in trials involving dendritic cell vaccination to try to identify factors in their pre-vaccination state that influence their propensity to make a T cell response to the vaccine.

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TUMOR IMMUNOLOGY AND ANTIGEN PROCESSING

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The central research theme of our group is the study of tumor antigens recognized by T lymphocytes. Besides our continued effort to identify additional antigens of interest, we mainly want to address a number of fundamental or mechanistic issues that have a direct impact on the utilization of such antigens as cancer vaccines in human patients. These antigens consist of peptides that are presented by MHC molecules at the cell surface and derive from intracellular proteins that are degraded by the proteasome. The intracellular pathway leading from the protein to the peptide/MHC complex is known as "antigen processing". We are currently studying the processing of several human tumor antigens by the proteasome, and we are particularly interested by the processing differences we have observed between the standard proteasome, which is present in most cells, and the immunoproteasome which is found in some dendritic cells and in cells exposed to interferon-gamma.

We are also studying a mouse preclinical model of cancer immunotherapy, where we try to define the optimal conditions to induce effective anti-tumor responses by various vaccination approaches against defined antigens. This led us to uncover a powerful mechanism of tumor resistance which is based on tryptophan catabolism by indoleamine-2,3 dioxygenase, an enzyme that we found frequently expressed in tumors. The resulting local tryptophan shortage appears to prevent the proliferation of lymphocytes at the tumor site. Inhibitors of indoleamine-2,3 dioxygenase are currently being tested in vivo for their ability to counteract this tumor resistance mechanism.

To obtain the most relevant information from such preclinical models, we are trying to build a new mouse melanoma model where tumors expressing a given antigen could be induced, using a transgenic system based on Cre-lox recombination. This should recapitulate the long-term host/tumor relationship that occurs in humans when a tumor slowly develops within a normal tissue.

Building on our expertise in antigen processing and presentation, we have also developed a collaboration with the Unité de Rhumatologie of the Cliniques Universitaires St-Luc to study antigen presentation by dendritic cells in Systemic Lupus Erythematosus (SLE), both in mouse models of SLE and in human patients.

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

Jacques Chapiro, Benoit Guillaume, Sandra Morel, Fanny Piette

Antigens recognized by cytolytic T lymphocytes (CTL), such as viral or tumor antigens, usually consist of peptides of 8-10 amino acids in length, which are presented by MHC class I molecules at the cell surface. Because such peptides derive from intracellular proteins, a processing step is required before they can be exposed to the cell surface in association with MHC molecules. Firstly, the peptide is produced, as a result of the degradation of the parental protein by the proteasome. Secondly, it is taken up by a dedicated transporter named TAP, and translocated inside the endoplasmic reticulum where it meets and associates with newly synthesized MHC molecules.

The first step of cleavage by the proteasome is crucial in that cleavage location determines the precise sequence of the final antigenic peptide. We have observed that this cleavage may occur differently in some cells, depending on their proteasome content. The proteasome comes in two forms : the standard proteasome, which is found in most cells, and the immunoproteasome, which is expressed by mature dendritic cells and by cells exposed to interferon-gamma (IFNγ).

We previously reported that a class-I restricted antigenic peptide derived from an ubiquitous human protein was processed efficiently by the standard proteasome but not by the immunoproteasome. As a result, the relevant epitope is not presented efficiently by mature dendritic cells, which contain immunoproteasomes (1). This could explain how certain potentially autoreactive CTL can escape tolerance induction in the thymus and fail to be activated in the periphery. We have now extended those observations to other epitopes derived from self proteins, and to several antigenic peptides of interest for cancer immunotherapy, including HLA-A2-restricted epitopes derived from tyrosinase, Melan-A^{MART1} and gp100. These results were obtained from in vitro experiments where synthetic peptides of about 20 amino acids, encompassing the epitope, are digested with highly purified preparations of either standard proteasomes or immunoproteasomes. The presence of the antigenic peptide in the digests is tested with the relevant CTL after pulsing on target cells (Fig. 1). It is then confirmed by HPLC and mass spectrometry. Consistent with their poor processing by the immunoproteasome, those epitopes are not presented efficiently to CTL by cells containing immunoproteasomes, such as tumor cells treated with IFNγ for 7 days, or cells transfected with cDNAs encoding the three immunoproteasome subunits, ß1i (LMP2), ß2i (MECL1), and ß5i (LMP7).

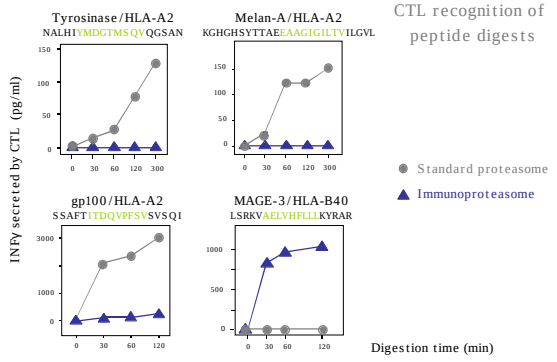

Fig.1. see text for explanations

On the contrary, we showed that another tumor epitope, which is derived from MAGE-3 and presented by HLA-B40, is processed by the immunoproteasome but not by the standard proteasome (Fig. 1). Accordingly, this epitope is presented to CTL only by tumor cells pre-treated with IFNγ (2).

Our observations indicate that the pool of antigenic peptides presented at the cell surface may differ substantially according to the proteasome type that is predominant in the cell. This may have major implications for immunotherapy, particularly for cancer immunotherapy, as it means that the peptide repertoire presented by tumor cells may differ from the repertoire presented by antigen-presenting cells (3). The peptide repertoire of tumor cells themselves may vary according to the localization of the tumor (e.g. primary tumor versus lymph node metastasis) and its level of exposure to IFNγ. Therefore, it appears essential for the success of cancer immunotherapy to study those processing differences in detail, so as to define the most effective vaccination strategy for each epitope and to use the appropriate combination of antigens in order to minimize the risk of tumor escape by proteasome switching.

Identification of new antigens recognized by autologous CTL on human melanoma

Wenbin Ma, Nathalie Vigneron (in collaboration with P. Coulie)

Melanoma line EB81 expresses several antigens recognized by autologous CTL. By using a cDNA expression cloning approach, we identified the antigens recognized by two of them. These antigens correspond to two distinct peptides derived from MAGE-C2, a gene with a cancer-germ-line expression pattern, which is expressed in about 40% of melanomas and 30% of bladder carcinomas. This gene had been isolated earlier by a genetic subtraction approach between tumor and normal material, and was not known yet to encode tumor antigens. Both peptides are presented by HLA-A2, which is the most frequent HLA allele in Caucasians. Because of their strict tumor-specificity and their wide expression in tumors, these new antigens represent promising targets for cancer immunotherapy.

Melanoma line LG2-MEL also expresses several antigens recognized by autologous CTLs. One of them consists of a peptide derived from tyrosinase and presented by HLA-B*3503. We have identified another antigen of LG2-MEL as a peptide presented by HLA-B*4403 and resulting from a point

mutation in gene OS-9 (4). This gene is expressed in various normal tissues. It is located on chromosome 12 in the vicinity of the CDK4 locus and is frequently co-amplified with CDK4 in human sarcomas. The mutation, a C-to-T transition, changes a proline residue into a leucine at position 446 of the OS-9 protein. Mutated transcripts were found in all the melanoma sublines of LG2-MEL. None of the 184 tumor samples collected from other cancer patients expressed the mutated transcript, indicating that this is a rare mutational event. Interestingly, some of the melanoma sublines of LG2-MEL have lost the wild-type allele of gene OS-9. Those sublines appear to grow faster in vitro than the sublines that retained the wild-type allele, suggesting that this loss of heterozygosity may favor tumor progression. The mutation we have identified in gene OS-9 might therefore participate in the oncogenic process by affecting the function of this potential tumor-suppressor gene.

TNF-mediated toxicity after massive induction of specific CD8+ T cells following immunization of mice with a tumor-specific peptide

Catherine Uyttenhove, Dominique Donckers, Luc Pilotte

In order to optimize the vaccination modalities applied in cancer immunotherapy trials, we have continued our systematic assessment of the efficacy of various immunogens to induce CTL responses in mice against defined tumor antigens. In the course of those studies, we immunized mice with antigenic peptide P815E, which is presented by H-2Kd and recognized by tumor-specific CTL raised against P815 tumor cells. This peptide is encoded by the ubiquitously expressed gene MsrA and carries a mutated residue conferring tumor specificity. Unexpectedly, we observed a severe toxicity occurring in the early hours after the third injection, resulting in the death of most mice within 24 h (5). The toxic syndrome was reminiscent of TNF-induced shock, and the sera of ill mice contained high levels of TNF. Toxicity was prevented by injection of neutralizing anti-TNF Abs, confirming the involvement of TNF. Depletion of CD8+ T cells could also prevent toxicity, and ex vivo experiments confirmed that CD8+ lymphocytes were the major cellular source of TNF in immunized mice. Tetramer analysis of the lymphocytes of immunized mice indicated a massive expansion of P815E-specific T cells, up to >60% of circulating CD8+ lymphocytes. A similar toxicity was observed after massive expansion of specific CD8+ T cells following immunization with another P815 peptide, which is encoded by gene P1A and was injected in a form covalently linked to an immunostimulatory peptide derived from IL-1. We conclude that the toxicity is caused by specific CD8+ lymphocytes, which are extensively amplified by peptide immunization in a QS21-based adjuvant and produce toxic levels of TNF upon further stimulation with the peptide. Our results suggest that immunotherapy trials involving new peptides should be pursued with caution and should include a careful monitoring of the T cell response.

A novel tumor immune escape mechanism based on tryptophan degradation by indoleamine 2,3 dioxygenase

Catherine Uyttenhove, Luc Pilotte, Ivan Théate, Dominique Donckers, Nicolas Parmentier, (in collaboration with Vincent Stroobants and Didier Colau)

It has been shown that T lymphocytes undergo proliferation arrest when exposed to tryptophan shortage, which can be provoked by indoleamine 2,3-dioxygenase (IDO), an enzyme that is expressed in placenta and catalyzes tryptophan degradation. Local tryptophan depletion by IDO expression has therefore been proposed as a natural immunosuppressive mechanism promoting tolerance of the fetus during pregnancy. Expression of IDO is also induced in many cells by interferon-gamma, and could thereby participate in the regulation of immune responses.

To determine whether tumors might use this mechanism to escape T-cell mediated immune responses, we measured the expression of IDO by RT-PCR in a series of murine and human tumor cell lines. We found that many lines were positive. Moreover, when we tested a large series of human tumor samples by immunohistochemistry with an IDO-specific antibody, we observed that a vast majority stained positive, including all prostatic, colorectal, pancreatic and cervical carcinomas.

Using the well-characterized model system of mouse tumor P815, where the antigen encoded by gene P1A is the major target of the tumor rejection response, we observed that expression of IDO by P815 tumor cells prevents their rejection by pre-immunized mice. This effect can be partly reverted by systemic treatment of mice with an inhibitor of IDO, in the absence of noticeable toxicity. These results suggest that the efficacy of therapeutic vaccination of cancer patients could be improved by concomitant administration of an IDO inhibitor.

Develoment of a mouse melanoma model for immunotherapy

Ivo Huijbers (in collaboration with Paul Krimperfort (NKI, Amsterdam) and Anne-Marie Schmitt-Verhulst (CIML, Marseille))

Immunotherapy represents an attractive approach for the treatment of cancer, in particular melanoma. Preclinical studies of various strategies of immunotherapy rely on model systems where tumor cells grown *in vitro* are inoculated into syngeneic mice. However, this does not recapitulate the long-term host-tumor relationship that occurs in patients during tumor development. In order to have a model system more relevant to the human situation, we are trying to develop a mouse strain in which we can induce melanomas expressing a tumor antigen of interest.

One of the most common site of genetic lesions in human melanoma is the *INK4A/ARF* locus, which encodes two distinct tumor suppressor proteins $p16^{INK4A}$ and $p14^{ARF}$. Genetic disruption of this locus predisposes mice to the formation of various tumor types, but is not sufficient to induce melanoma unless the Ras-pathway is specifically activated in melanocytes. In order to have a fully controlled model system for melanoma, we planned to generate transgenic mice in which the deletion of the *Ink4a/Arf* genes and the melanocyte-specific expression of both activated Harvey-Ras^{G12V} and a well characterized antigen is spatially and temporally regulated by a fusion protein between the Cre-recombinase and the tamoxifen responsive hormone-binding domain of the estrogen receptor ($CreER^{\Delta D}$). The antigen is encoded by *P1A*, a gene expressed in several tumors but silent in normal tissues except testis and placenta. The tumor induction in these mice will be performed by topical administration of tamoxifen, which should be sufficient to induce the essential genetic rearrangements in melanocytes necessary to establish neoplastic transformation.

Six transgenic lines were generated, harboring a construct of respectively the tyrosinase promoter with two enhancer elements, a $CreER^{\Delta D}$ fusion gene flanked by loxP sites, a V12 mutated H-Ras gene, an internal ribosomal entry site and the P815A-antigen encoding gene, P1A. The expression and regulation of the $CreER^{\Delta D}$ fusion gene was analyzed by crossing these mice to a Rosa26 Cre reporter strain. In three transgenic lines specific blue staining could be observed in melanocytes after topical treatment of the ear by 4-hydroxytamoxifen. This implied that the $CreER^{\Delta D}$ fusion gene was expressed specifically in melanocytes and activated upon treatment with its ligand. In order to determine whether the transgene was still intact and functional after Cre-recombination, these transgenic lines were crossed with a CMV-Cre-deleter strain. In one transgenic line a break in the $CreER^{\Delta D}$ gene was observed after recombination. In the other two lines, H-ras and P1A were still detected after Cre-recombination implying that the transgene was arranged in such a way that the tyrosinase-promoter was now driving the expression of genes H-ras and P1A. These lines are now being crossed to a homozygous conditional *Ink4a/Arf* knock-out background in order to induce melanoma formation by applying 4-hydroxytamoxifen to the skin.

Antigen presentation by dendritic cells in Systemic Lupus Erythematosus

Bernard Lauwerys (in collaboration with Frédéric Houssiau, Unité de Rhumatologie)

Systemic Lupus Erythematosus (SLE) is an autoimmune disorder characterized by overt polyclonal B cell activation and autoantibody production against nuclear antigens. We are studying the involvement of dendritic cells in the impaired central and peripheral tolerance which is characteristic of the disease. Studying strains of mice that are congenic for different SLE susceptibility loci and were developed by E. K. Wakeland (University of Texas), we observed that dendritic cells from one of these strains (Sle3) are characterized by an increased gene expression of BAFF, a cytokine that promotes B cell survival and activation. The role of these dendritic cells in the induction of antibody production is currently under investigation. Another aspect will be a detailed analysis of the antigen presentation capacity of dendritic cells from BWF1 mice, which also develop SLE. Besides the expression of surface markers, we will investigate the capacity of those dendritic cells to induce central tolerance in the thymus by negative selection, using P1A-TCR transgenic mice that have been developed within the group.

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GENES EXPRESSED IN CANCER AND GERMLINE CELLS

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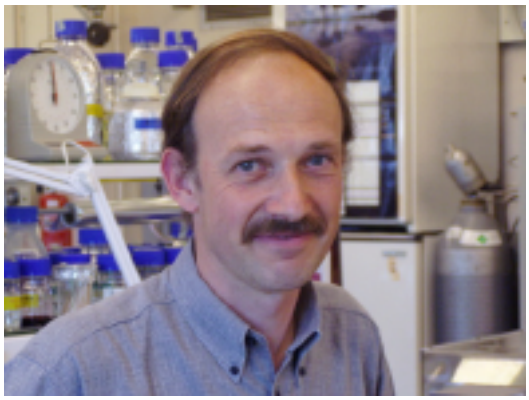

Human tumors express specific antigens arising from the activation of genes, such as MAGE genes, that are normally expressed only in germ cells. As germ cells are not subject to scrutiny by the immune system, antigens encoded by these genes are strictly tumor-specific. We are trying to identify new genes that present the same pattern of expression as MAGE genes. Additionally, efforts are devoted to determining both the function of "cancer-germline" genes, and the mechanisms leading to their activation in tumors.

Screening procedures based on differential expression profiling allowed the isolation of several genes with cancer and germline specific expression (1 -5). Most of these genes have their normal site of expression in spermatogonia, the pre-meiotic stage of sperm development, and are located on the X chromosome (Fig. 1). The spermatogonia-restricted expression of these genes is likely imposed by their chromosomal location, as the X chromosome becomes inactivated at the onset of meiosis in sperm cells.

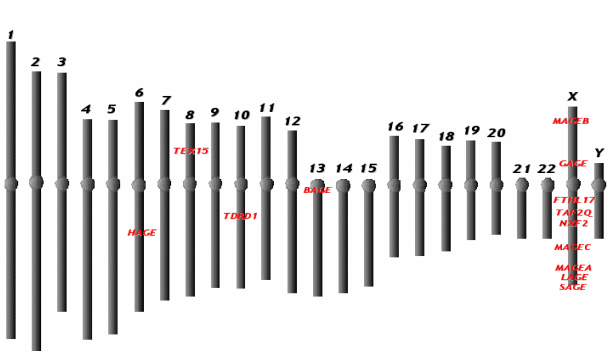

Fig. 1. Chromosome mapping of "cancer-germline" genes

MAGE-A1 belongs to a family of twelve genes located on the X chromosome in region q28 (6-7). To analyze the functions of MAGE-A1, we searched to identify protein partners of this protein. Using yeast two-hybrid screening, we found interaction between MAGE-A1 and transcriptional regulator SKIP. SKIP is an adaptor protein that connects DNA-binding proteins such as Smad3, the Vitamin D receptor, CBF1 or MyoD, to proteins that activate or repress transcription. A repression complex including histone deacetylases (HDAC) is known to bind to SKIP and CBF1. In the presence of the intracellular part of Notch1 (Notch1-IC), the repression complex is detached from SKIP by Notch1-IC and recruitment of an activation complex including histone acetyltransferases (HAT) is facilitated by SKIP. To examine whether MAGE-A1 could interfere with a signalling pathway involving SKIP, we expressed MAGE-A1 in mammalian cells in which Notch1-IC binds to SKIP fused to the Gal4 DNA-binding domain and activates a *CAT* reporter gene containing Gal4-binding sites near its promoter (Fig. 2). We found that MAGE-A1 inhibited the Notch-1/SKIP transcriptional activation. Deletion analysis indicate that binding to SKIP is required to observe MAGE-A1-mediated repression of Notch1-IC transactivation. Moreover, MAGE-A1 was found to actively repress transcription by binding and recruiting histone deacetylase 1. Our results suggest that by binding to SKIP and by recruiting histone deacetylases, MAGE-A1 protein present in the nucleus could repress genes implicated in development and spermatogenesis. We are now trying to identify the genes that are regulated by MAGE-1 by using an inducible transfected *MAGE-A1* gene and the microarray technology.

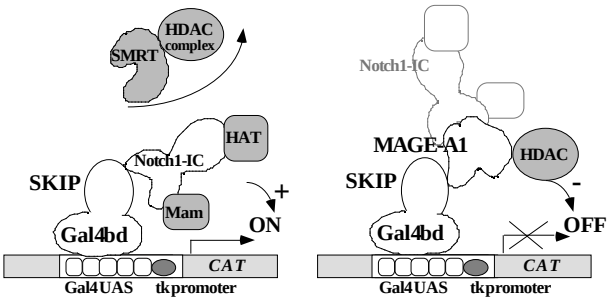

Fig. 2. MAGE-A1 counteracts Notch1-IC transactivation and recruits histone deacetylases

Studies on the transcriptional regulation of cancer-germline genes, such as *MAGE-A1*, showed that DNA methylation is an essential component of their repression in normal somatic tissues (8). The promoters of these genes contain a high density of CpGs, but unlike classical CpG-rich promoters they are heavily methylated in all somatic tissues. In constrast, they are unmethylated in germ cells and in tumors that express these genes. Demethylation and therefore activation of cancer-germline genes in tumors was found to be coincident with overall genome demethylation, a process known to occur in many cancers (9-10). We are currently studying the mechanisms of demethylation of these genes in tumors. This should give insight into the processes leading to genome hypomethylation in cancers. It may also help designing procedures to induce the expressionof specific antigens on tumors, thereby facilitating their elimination by the immune system.

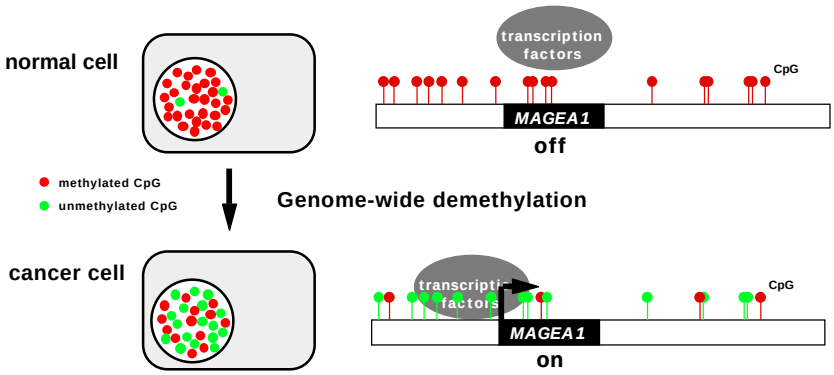

Fig. 3. *MAGEA1* activation as a result of genome demethylation in tumors

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IDENTIFICATION OF HUMAN TUMOR ANTIGENS

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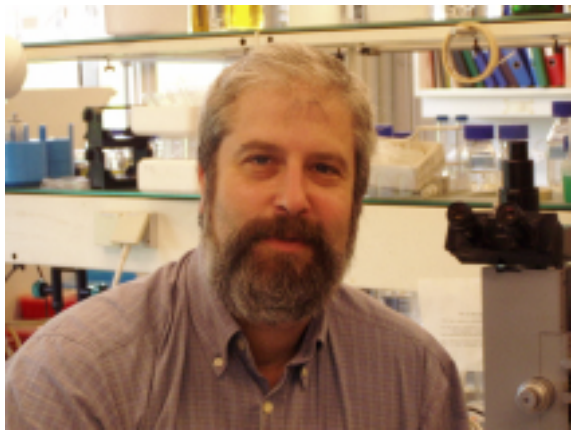

The group led by Pierre van der Bruggen is defining antigenic peptides encoded by genes such as those of the MAGE family. Therapeutic vaccination of cancer patients with MAGE peptides is now in progress, and the identification of additional antigenic peptides is important to increase the range of patients eligible for therapy and to provide tools for a reliable monitoring of the immune response. The group is also involved in designing reliable methods for the monitoring of the CD4$^+$ T cell response to cancer vaccines, and in the study of functional defects of T cells.

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

Yi Zhang, Vincent Stroobant, Christophe Panichelli, Sabrina Ottaviani, Tetsuto Kobayashi

"Cancer germline" genes such as those of the MAGE family are expressed in many tumors and in male germline cells, but are silent in normal tissues. They encode shared tumor specific antigens, which have been used in therapeutic vaccination trials of cancer patients. The first antigens and genes that code for these antigens were identified with anti-tumor cytolytic T lymphocytes obtained from cancer patients (1). A few HLA class I-restricted antigenic peptides were identified by this "direct approach" (2). A large set of additional cancer-germline genes have now been identified by purely genetic approaches (3, 4). As a result, a vast number of sequences are known that can code for tumor-specific shared antigens, but most of the encoded antigenic peptides have not been identified yet. The identification of a large number of antigenic peptides presented by HLA class I and class II molecules is likely to be important for the future of clinical trials with defined antigenic peptides. A large set of peptide/HLA combinations will alleviate HLA restriction and widen the set of eligible patients. It will also facilitate the design of concurrent immunizations against several antigens. Such immunizations could increase the primary anti-tumor efficacy of the vaccine and also decrease the risk of tumor escape by loss of antigen expression.

We have used approaches that we have loosely named "reverse immunology" (5). They aim at identifying antigenic peptides recognized by T cells, using gene sequences as starting point. We have focused this search on the cancer-germline genes, which are expected to code for tumor-specific shared antigens on the basis of their pattern of expression.

Search for antigenic peptides recognized by CD8$^+$ T cells

We stimulated CD8$^+$ T lymphocytes with dendritic cells transduced with viral vectors containing complete MAGE-coding sequences. As this requires the processing of the antigen by the dendritic cells, we surmised that the peptides that would be identified would also be processed in the tumors expressing the MAGE genes. A difficulty of the use of recombinant viruses is the activation of CTL precursors directed against viral antigens. We circumvented this problem by using different vectors for the stimulation of the microcultures, for the lytic assay with the responder T cells, and for the cloning step. This procedure is summarized in Figure 1. Dendritic cells were infected with either an adenovirus or a canarypoxvirus and they were

used to stimulate microcultures of autologous CD8$^+$ T lymphocytes (6). After three weekly stimulations, the responder cells were tested for lysis on autologous EBV-B cells infected with vaccinia-MAGE. Positive microcultures were cloned. To identify the antigenic peptide, the resulting CTL clone was tested for lysis of autologous EBV-B cells pulsed with a complete set of peptides of 16 amino acids that overlap by 12. When a peptide scored positive, shorter peptides were synthesized to identify the shortest optimal peptide. To identify the HLA presenting molecule, the CTL clones were tested for stimulation by cells transfected with the MAGE cDNA together with cDNAs coding for the possible HLA presenting molecules. Finally, relevant tumor targets were used in a lysis assay to ascertain that the antigen was also processed by tumor cells.

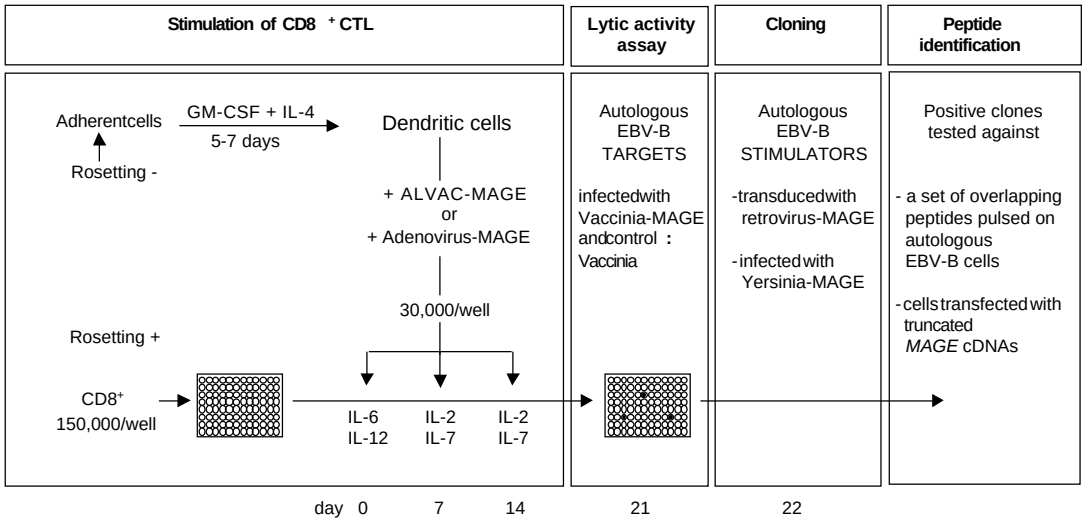

Fig. 1. Overview of the procedure to obtain anti-MAGE CD8$^+$ CTL clones by stimulation with dendritic cells infected with viral vectors carrying a MAGE coding sequence

We have listed in a database class I-restricted antigenic peptides that are encoded by cancer-germline genes (http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm). MAGE-1 and MAGE-3 antigenic peptides are available for more than 90% of Caucasians.

We have identified a MAGE-3.B40 antigen which is the first example of a tumor-specific antigen exclusively presented by tumor cells expressing the immunoproteasome (7). This work was done in collaboration with the group of Benoît Van den Eynde.

We have found that MAGE-1 peptide SAYGEPRKL is recognized by CTL clones that are restricted either by HLA-Cw3, Cw6 or Cw16 (5). The presentation of the same peptide by different HLA molecules may be frequent for HLA-C molecules, because they are more closely related to each other in the peptide-binding region than HLA-A and B molecules. But for HLA-A and B also this may occur more frequently than usually thought. MAGE-3 peptide MEVDPIGLY is presented to different CTL by HLA-B*4402, B*4403, and B*1801 (5). MAGE-1 peptide EADPTGHSY was found to be recognized by different CTL on HLA-A1 and B35 molecules, and to bind to HLA-A29 (5). The same was found for the MAGE-3 homologous peptide EVDPIGHLY.

These results have consequences for the monitoring of the immune response of patients vaccinated with such tumor-specific shared peptides. A number of HLA-A1 patients were injected with MAGE-3.A1 peptide EVDPIGHLY, at a time when we did not know that it could be presented by B35 and A29 (5). The immune response was evaluated with HLA-A1 tetramers folded with the MAGE-3 peptide. Thus, A29 or B35-restricted responses against the peptide may have been missed.

Search for antigenic peptides recognized by CD4$^+$ T cells

Studies in several animal models have demonstrated an important role for CD4$^+$ T cells in inducing and maintaining anti-tumor immunity. It is therefore possible that the addition of antigenic peptides presented by class II to those presented by class I will improve the efficacy of therapeutic anti-tumor vaccination. To identify new HLA-peptide combinations, we used dendritic cells loaded with a recombinant MAGE protein to stimulate autologous CD4$^+$ T lymphocytes (8). After four weekly stimulations, the responder cells were tested for their ability to secrete IFN-γ upon stimulation with the antigen, and the positive microcultures were cloned. The procedure is summarized in Figure 2.

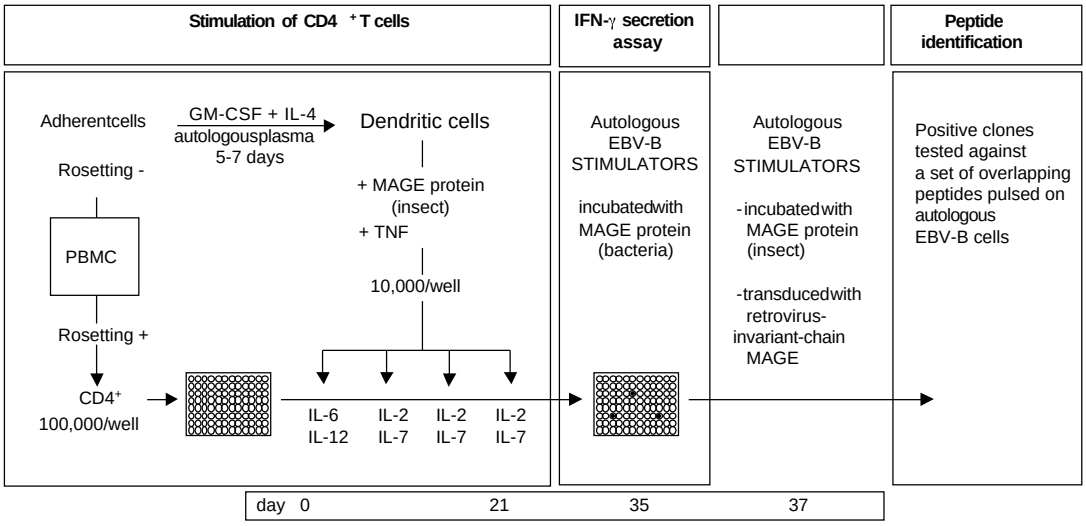

Fig. 2. Overview of the procedure to obtain anti-MAGE CD4$^+$ T cell clones by stimulation with dendritic cells loaded with a whole protein

To identify the antigenic peptide, the positive clones were stimulated with a set of peptides of 16 amino acids that overlapped by 12 and covered the entire MAGE protein sequence. The positive peptide was then tested for recognition on several Epstein-Barr virus immortalized B cell lines (EBV-B cell lines) to identify the HLA presenting molecule. Because a large number of the CD4$^+$ T cells that were obtained in our first experiments appeared to be directed against bacterial contaminants, we chose to alternate the sources of protein used at the various stages of the procedure. For example, to stimulate the lymphocytes, we used a MAGE protein produced in insect cells, and to test the specificity of the responder lymphocytes, we used a protein produced in bacteria. Microcultures that specifically produced IFN-γ after stimulation with the MAGE protein were cloned by limiting dilution using autologous EBV-B stimulator cells either loaded with the MAGE

protein used during the stimulation step, or transduced with a retroviral construct encoding a truncated human invariant chain (Ii) fused with the MAGE protein.

MAGE-1 and MAGE-3 antigenic peptides identified by this procedure are listed in a database (http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm). They include a MAGE-3 peptide presented by HLA-DP4, which is expressed by more than 70% of Caucasians (9). This peptide could not have been found by a peptide stimulation approach because no consensus anchor residue was known for HLA-DP4.

The normal anti-MAGE-3.A1 repertoire

Christophe Lonchay

To estimate the frequency of anti-MAGE-3.A1 CTLp directed against a MAGE-3 peptide (EVDPIGHLY) presented by HLA-A1 in blood from normal donors, groups of about 200,000 purified CD8 cells were stimulated with autologous PHA-activated T cells incubated with peptide MAGE-3.A1, in the presence of IL-6 and IL-12 during the first week followed by two weekly restimulations in the presence of IL-2 and IL-7. After the third week the cultures were tested in a lysis assay against HLA-A1 peptide-pulsed EBV-B cells. On the basis of the fraction of positive groups, the frequency of CTLp directed against the MAGE-3.A1 antigen was estimated to be about 2×10^{-7} in the CD8 lymphocyte population.

Another estimate was obtained recently for an hemochromatosis patient, from whom a large number of blood lymphocytes was collected. Purified blood T cells were incubated with an A1/MAGE-3 tetramer. The very rare cells that were stained by the tetramer and also by anti-CD8 antibodies were sorted at one cell per well and restimulated with irradiated HLA-A1 cells pulsed with the MAGE-3.A1 peptide. On the basis of the number of T cell clones that multiplied and were stained by the tetramer, we estimated the frequency of the naive anti-MAGE-3.A1 CTLp at 6×10^{-7} of the CD8 T cells for. With about five liters of blood containing about 10^6 PBMC/ml with about 15% CD8 T cells, and about 2% of total lymphocytes being located in the blood, the body total number of CD8 cells is approximately 4×10^{10}. Therefore a blood frequency of 3×10^{-7} of the CD8 corresponds to 32,000 anti-MAGE-3.A1 precursors. In the course of this analysis, 15 anti-MAGE-3.A1 T cell clones were obtained, corresponding to 14 different T cell receptors (TCR). Using these numbers to estimate the diversity of the naive T cell repertoire against MAGE-3.A1, one can state with 90% certainty that the naive repertoire directed against MAGE-3.A1 consists of 40 to 400 different TCR.

A reversible functional defect of $CD8^+$ T lymphocytes involving loss of tetramer labeling

Nathalie Demotte

We have observed that human CTL clones lose their specific cytolytic activity and cytokine production under certain stimulation conditions, while retaining an antigen-dependent growth pattern. These inactive CTL simultaneously lose their labeling by an HLA-peptide tetramer, even though the amount of TCR-CD3 at their surface is not reduced (10). The tetramer-negative cells recover tetramer staining and cytolytic activity after stimulation with tumor cells in the presence of a supernatant of activated lymphocytes. Our results suggest the existence of a new type of functional defect of CTL. They also indicate that tetramers may fail to reveal some CTL bearing the relevant TCR, even when such functionally arrested CTL retain the potential to participate in immune responses because their defect is reversible. We will analyze the ability of CTL to be labelled by tetramer during the days following the antigenic stimulation. We also plan to analyze the composition of lipid rafts of tetramer-positive and tetramer-negative cells. In addition, we will analyze tetramer-positive and tetramer-negative cells for differential expression of genes by microarray analyzes.

Detection of anti-vaccine CD4 T cell response in vaccinated patients

Yi Zhang

Patients injected with class II-restricted peptides

For therapeutic vaccination trials, the vaccine can consist of defined antigenic peptides. This approach greatly facilitates the monitoring of the T cell response, because the presumed target of the T cells is completely defined. It allows the use of HLA-peptide tetramers to detect T cell responses in patients. HLA class II tetramers have been more difficult to obtain than HLA class I tetramers, but have already been used to detect low frequencies of CD4 T cells. Didier Colau has recently succeeded in obtaining a DP4.MAGE-3 tetramer, which was produced in insect cells. It stained specifically relevant CD4 clones and we are using this tetramer to estimate the frequency of anti-vaccine CD4 T cells in patients injected with either the MAGE-3.DP4 peptide or dendritic cells pulsed with this peptide.

Patients injected with a protein

For therapeutic vaccination trials, the vaccine can also consist of the entire protein. This has the advantage that antigenic peptides binding to a broad set of HLA molecules can be processed from the vaccine, so that the patients do not have to be selected according to their HLA. However, the detection of the anti-vaccine T cells is more laborious and can not rely on the use of HLA-peptide tetramers. An interesting alternative assay uses bispecific antibodies that bind to the cell surface and capture cytokines immediately after their production. The cells are kept alive, can be cloned, and analyzed further for specificity and TCR expression. We have succeeded to detect anti-MAGE specific T cells using antigen-presenting cells pulsed with a peptide as stimulators. We are now trying to use dendritic cells loaded with a MAGE-3 protein so as to be able to monitor the immune response of patients vaccinated with an entire protein.

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THERAPEUTIC VACCINATION OF CANCER PATIENTS WITH TUMOR-SPECIFIC ANTIGENS

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Tumor cells carry antigens such as MAGE antigens that are absent from normal tissues, and that can be targeted by cytolytic T lymphocytes (CTL) (1). While it is possible to make such CTL recognize and kill autologous tumor cells in vitro, the precise way to induce an effective CTL response against a MAGE antigen in cancer patients is not known yet. In clinical vaccination trials, patients with a MAGE expressing cancer, often melanoma, are treated repeatedly with a MAGE vaccine. These trials have two main objectives. First, the effectiveness of various vaccination modalities can be assessed by following the clinical evolution of the tumor, by analyzing whether a specific CTL response to the vaccine antigen occurred, and by determining whether immunological and clinical responses are correlated. Secondly, T lymphocytes and tumor samples collected at different timepoints during vaccination can be analyzed in detail, which improves our understanding on what happens in patients who experience regression of metastatic lesions, and which may explain why this does not happen in the majority of patients with overall disease progression. This knowledge can then be used to design new vaccination modalities.

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 330 patients have been included in these multicentric trials.

Current status of the trials

Twenty-one melanoma patients included in the ongoing study LUD 97-004 have received 9 immunizations with the MAGE-3.A1 peptide, injected intradermally (ID) and subcutaneously (SC) every 10-11 days. Tumor regression was observed in three 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 (2), the increase in the vaccination frequency does not seem to improve the clinical benefit. Using in vitro PBL stimulation in limiting dilution conditions followed by HLA/peptide tetramer staining, anti-MAGE-3.A1 CTL responses were detected in 1 of 2 patients with regression, and in none of 4 patients with disease progression. Seven additional patients have received the same peptide associated with the MAGE-3.DP4 peptide, in order to induce simultaneous CD4+ T lymphocyte responses. No regressions were observed, suggesting that no clinical benefit is obtained by the addition of this HLA class II restricted peptide. In a future clinical trial, the MAGE-3.A1 peptide will be mixed with an immunostimulatory CpG-containing oligonucleotide to increase its immunogenicity. In another trial, we will test whether a combination of 4 peptides (MAGE-3.A1, NA17.A2, tyrosinase.A2 and Melan-A.A2) that are individually associated with regression of melanoma metastases will improve the tumor response rate.

The clinical efficacy of the MAGE-3 protein injected ID and SC without adjuvant in non-visceral melanoma patients was tested in study LUD 99-003. Patients received 300 µg of MAGE-3 protein

on 6 occasions at 3-week intervals. To date, 5 out of 26 evaluable patients have shown regressions, including 1 complete response lasting for more than 1 year. 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.We will now mix this recombinant protein with adjuvant AS15 containing an immunostimulatory CpG nucleotide, and combine these IM injections with the administration of selected class I or class II peptides by ID and SC routes, which may result in the simultaneous activation of both CD8+ and CD4+ specific T lymphocytes (Study LUD 02-002). Moreover, since a patient with metastatic bladder cancer experienced regression of lymph node metastases upon immunization with the MAGE-3 protein mixed with the adjuvant SB AS-2 (3), simultaneous administration of a MAGE recombinant protein and of some corresponding MAGE peptides will also be tested in the neo-adjuvant setting, in patients with bladder cancer (Study LUD 01-013).

In the LUD 97-005 trial, 40 patients with advanced cancer, including 37 with melanoma, were vaccinated with a recombinant canarypox (ALVAC) virus containing a minigene that encodes the MAGE-1.A1 and MAGE-3.A1 antigens, followed by booster vaccinations with the 2 corresponding peptides. The treatment comprised 4 ALVAC injections followed by 3 peptides injections, all ID and SC, separated by 3 weeks each. Local inflammatory reactions at the sites of ALVAC injection were common, but were moderate in intensity and transient in duration. Among the 30 melanoma patients who received at least 4 ALVAC vaccinations, six experienced regression of one or more melanoma metastases. Significant CTL responses were detected in 4 of the 6 patients with regressions, and in 2 of 11 patients with disease progression using our tetramer assay. We plan to investigate in a new trial whether increasing the dose of ALVAC would result in improved immunological and clinical responses.

In study LUD 01-006, patients with completely resected primary or regional metastatic melanoma with a high risk of relapse have been vaccinated with either the MAGE-3.A1 or MAGE-10.A2 peptide injected ID and SC every 2 weeks on 6 occasions. The objective of this ongoing trial is to analyze whether vaccination of melanoma patients with less advanced disease in the adjuvant setting improves the immunological response to a peptide vaccine. Up to now, no CTL response has been detected by our tetramer assay in the 13 patients who have received the complete treatment, including 7 patients with a resected tumor that did not express the appropriate antigen and who are assumed to be immunologically naive.

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, whatever the MAGE vaccine used. About 10% of the patients show complete or partial clinical responses. Some of them lasted for several years (4). This frequency is far beyond the reported incidence of spontaneous regressions of melanoma metastases, estimated at 0.2-0.3%, indicating that these regressions are linked to the vaccinations.

CTL responses can be detected in a minority of patients vaccinated either with peptide or ALVAC virus. The responses appear to be weak and are mainly monoclonal. The relative frequency of CTL responders versus non-responders is higher in patients who had tumor regressions (5). However, more patients need to be analyzed before statistically significant conclusions can be drawn.

No increased immunogenicity of peptide vaccines was observed in disease-free patients with less advanced melanoma, as compared with patients with active, antigen-bearing metastases.

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ANALYSIS OF T CELL RESPONSES OF VACCINATED CANCER PATIENTS *

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The identification of antigens recognized on human tumors by autologous T lymphocytes has opened the way for therapeutic vaccination strategies involving defined tumor antigens such as the MAGE antigens (1, 2). Tumor regressions have been observed in a minority of treated melanoma patients. Such clinical responder patients have been found following immunization with peptides and recombinant ALVAC viruses. In some patients, a monoclonal T cell response was observed and the level of the response appeared to be stable during the course of the vaccination protocol (3, 4). Since detectable T cell responses occured more frequently in patients who show signs of tumor regression than in those who did not, we consider the possibility that the limiting factor for the anti-tumor effect of the vaccine is the intensity of the CTL response to this vaccine (5, 6). Improving the efficacy of such vaccines would thus critically depend on their capacity to trigger a robust immune response. A novel approach to vaccination is to exploit the potentiality of dendritic cells that are widely accepted to be particularly effective in presenting antigens to T cells and immunize cancer patients with a sample of their autologous dendritic cells charged with tumor antigens. We initiated a collaboration with G. Schuler and B. Schuler-Thurner at the University of Erlangen (Germany), who vaccinated advanced stage IV melanoma patients with mature, monocyte-derived dendritic cells pulsed with MAGE peptides and observed regression of some metastases.

Methods for evaluation of T-cell responses in vaccinated cancer patients

To establish whether there is a correlation between tumoral regressions and T-cell responses against the vaccine antigen, we evaluated the responses of patients vaccinated with a MAGE-3 antigenic peptide, with a recombinant virus coding for this peptide or with MAGE peptide-pulsed dentritic cells.

The recent development of tetrameric peptide-MHC class I and class II complexes (tetramers) allows the direct identification of antigen-specific T-cells. The technology has been introduced and is still being developed by Didier Colau..

To detect low-level responses, blood lymphocyte microcultures were stimulated with the antigenic peptide in limiting dilution conditions (Mixed Lymphocyte Peptide Culture, MLPC), followed by tetramer analysis, cloning of the tetramer-positive cells, and T-cell receptor (TCR) sequence analysis of the cytolytic T lymphocyte (CTL) clones that showed strict specificity for the vaccine antigen (for method, see P. Coulie, ICP report).

Christophe Lurquin and Bernard Lethé focused their efforts on detailed analysis of frequencies of characterized T-cell clones in blood, metastases and non tumoral tissue samples, using ‘clonotypic’ polymerase chain reaction (PCR) amplifications specific for the Vα and Vβ rearrangements of relevant TCR. These PCR amplifications on cDNA were sensitive enough to detect one CTL expressing a given TCR mixed with $3x10^7$ PBMC

of normal donor and they were highly specific for the given TCR insofar as no product was amplified with cDNA prepared from $3x10^7$ PBMC of 5 unrelated donors.

A melanoma patient vaccinated with a tumor-specific antigenic peptide encoded by gene MAGE-3

A monoclonal CTL response against a MAGE-3 antigen presented by HLA-A1 was observed by the *in vitro* tetramer analysis in a melanoma patient who showed partial rejection of a large metastasis after treatment with a vaccine containing only the tumor-specific antigenic peptide (patient CP64, see P. Coulie).

Since all the $CD8^+$ blood lymphocytes directed against the vaccine antigen presented the same TCR, quantitative evaluation of the overall anti-MAGE-3 CTL response could be easily carried out on blood lymphocytes by reverse transcription-PCR amplification (RT-PCR). This study indicated a frequency of T cells expressing the TCR 48 of 1/20,000 among $CD8^+$ cells in two postimmunisation blood samples collected respectively 5 and 6 months after the onset of vaccination, whereas this TCR was not found among $2.5x10^6$ $CD8^+$ in blood collected before vaccination (table 1).

These results prove that the vaccination induced at least a 100-fold amplification of anti-MAGE-3.A1 CTL clone 48 and that vaccines containing only a tumor-specific antigenic peptide can elicit a CTL response (3).

We have also examined a non-regressing metastasis for the presence of CTL 48. Sections that were 7 μm thick and ~1 cm in diameter were divided in 12 smaller regions and the RNA of each of them was tested by the clonotypic RT-PCR amplification of TCR 48. An average of 3/12 positive areas was obtained. The analysis of immediately adjacent sections showed that there was no correlation between the location of the positive areas in different sections, suggesting that the positives represent single cells. By using CD8β immunostaining on adjacent sections to evaluate the number of $CD8^+$ T cells, we obtained a frequency of ~1/5,000 $CD8^+$ for CTL 48 in this nonregressing metastasis, without significant difference with the postimmune blood frequency.

Our experiments also suggest that the absence of immune attack on this metastasis is not due to a lack of expression of the MAGE-3.A1 antigen by the tumor cells since the melanoma cells were stained by a conformational antibody directed against functional MHC class I molecules and expressed MAGE-3 and HLA-A1 genes as demonstrated by RT-PCR.

Table 1.Detection of TCR 48 by RT-PCR in groups of PBMCs

PBMCs	Number of PBMCs/group	Proportion of $CD8^+$	Positive groups/ Tested groups	Frequency (among $CD8^+$)
Preimmunization	10^7	5%	0/5	$<4x10^{-7}$
Postimmunization I	$7.5x10^4$	6%	4/20	$5x10^{-5}$
Postimmunization II	10^5	9%	11/30	$5x10^{-5}$

A melanoma patient vaccinated with a MAGE-3 recombinant canarypox virus

Patient EB81 had about 70 cutaneous metastases when she was vaccinated with a recombinant canarypox virus of the ALVAC type containing a minigene encoding the MAGE-3.A1 peptide. Repeated injections of ALVAC were followed by vaccinations with the MAGE-3 peptide. Ten months after the first vaccine, all the metastases had become undetectable except an enlarged lymph node which was resected. A similar vaccination protocol alternating virus and peptide injections was carried on during two years and the patient was still free of detectable melanoma.

Blood lymphocytes collected before vaccination were analyzed with the MLPC-tetramer method. No anti-MAGE-3 CTL could be detected among 10^7 $CD8^+$ cells, suggesting a frequency similar to that found in normal donors (4). Two different anti-MAGE-3.A1 CTL clones were identified in postimmune blood : CTL 35 found in more than 95% of the independent microcultures which were analyzed and CTL 103, suggesting that this MAGE-3.A1 response was essentially monoclonal.

PCR amplifications specific for the TCR 35 Vα and Vβ rearrangements were applied directly to cDNA obtained from groups of PBMC. In the first post-ALVAC sample, the frequency of CTL C35 rose to 3.6 x 10^{-6} of the $CD8^+$, suggesting that the ALVAC vaccination induced at least a 30 times amplification of anti-MAGE-3.A1 T cells. The blood frequency of CTL 35 stayed between 1.5 x 10^{-6} and 3.3 x 10^{-6} during one year of vaccination with peptide. Afterwards, a set of five weekly injections of MAGE-3.A1 peptide given therefore at short intervals did not increase the frequency (1.4 x 10^{-6}) whereas four weekly injections of recombinant ALVAC given five months later boosted the anti-MAGE-3.A1 response with a frequency of CTL 35 increasing to ~1/75,000 $CD8^+$. The frequency did not change signifiantly after new series of peptide and virus injections : values ranging from 4.7 x 10^{-6} after a boost with peptide to 1.1 x 10^{-5} after injections of ALVAC were observed by the clonotypic RT-PCR amplification of TCR 35 (4).

By stimulating blood lymphocytes from melanoma patient EB81 with autologous tumor cells (MLTC), a series of CTL clones that specifically lysed autologous melanoma cells were isolated. Some of these CTL clones appeared to recognize peptides presented by HLA-A2 and encoded by gene MAGE-C2, an other cancer-germline gene which is expressed at high level by the melanoma cells of patient EB81. We analyzed the TCR Vβ gene expression of this set of anti-MAGE-C2 CTL clones. One of them, named CTL 16, seemed to be amplified after vaccination since it was retrieved many times as independent clones from the postimmune blood but was not found in preimmune blood samples. Clonotypic RT-PCR amplifications of TCR 16 Vα and Vβ rearrangements indicated an average frequency of the corresponding CTL in blood throughout the whole time of vaccination of ~3.5 x 10^{-5} among the $CD8^+$.

To investigate the involvement of the MAGE-C2-specific CTL in the tumor regression process observed in this patient after vaccination against a MAGE-3 antigen, we have analyzed the frequencies of the anti-MAGE-3.A1 CTL 35 and the anti-MAGE-C2.A2 CTL 16 in the resected metastatic lymph node which presented histological signs of regression. Sections of 7 µm thick and ~80 mm^2, fragments of such sections and groups of cells excised from sections with laser microdissection were tested by RT-PCR for the presence of TCR 35 and TCR 16. We obtained a frequency of ~1/32,500 CD8 for anti-MAGE-3 CTL, which represents a 20-fold enrichment relative to blood frequency at the time of the metastasis resection. Moreover, the anti-MAGE-C2 CTL was at least 275-times enriched in the resected sample with a frequency higher than 1/100 CD8 in the lymph node tissue and a frequency >1/5 CD8 in tissue regions strongly invaded by tumoral cells (Figure 1).

Our results suggest that tumor-specific CTL others than those directing against the vaccine antigen can be stimulated after vaccination and may have a function in the tumor rejection process that follows vaccination.

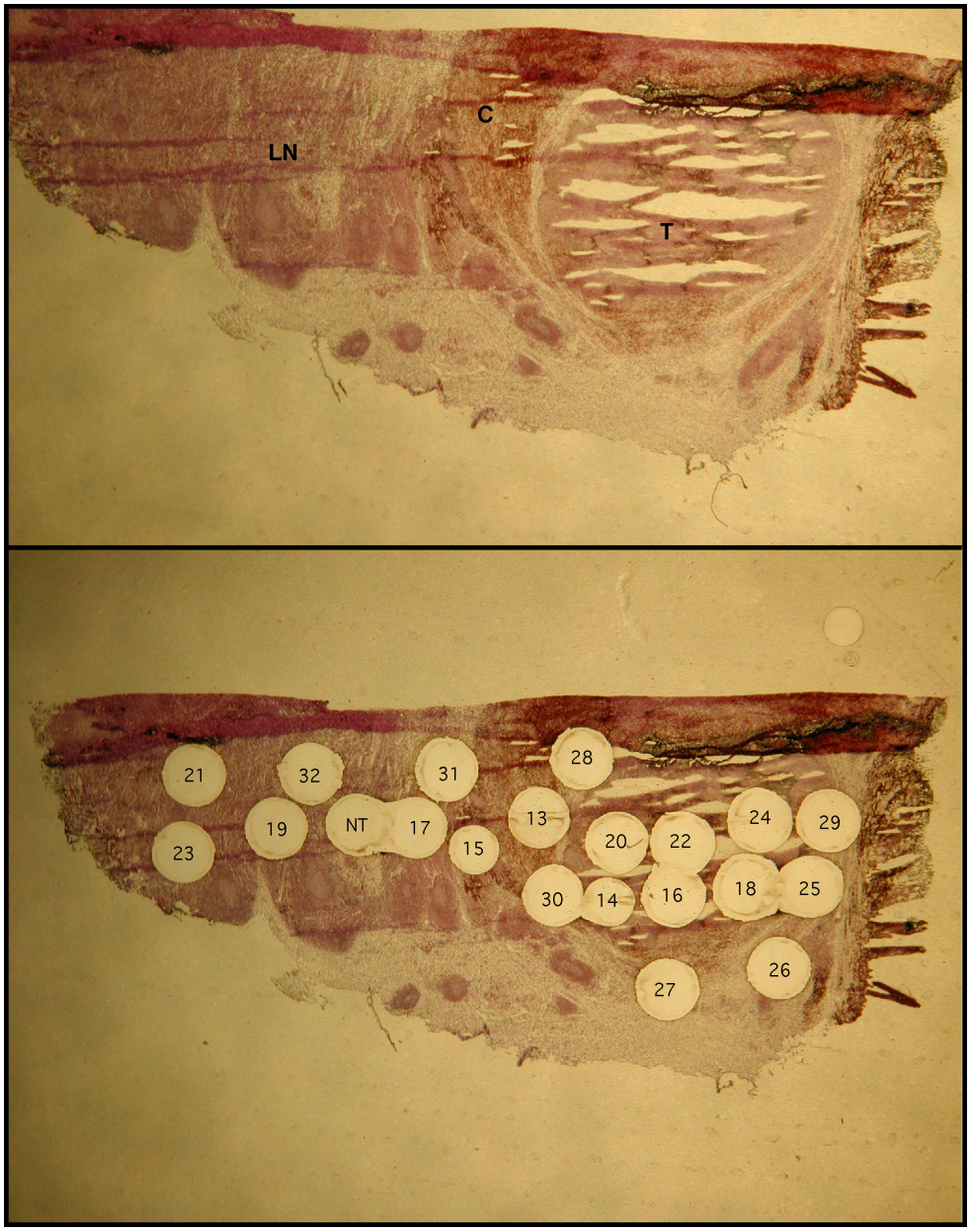

Fig. 1. Section of invaded lymph node from patient EB81
Upper panel : hematoxylin-stained cryosection before laser microdissection ; T, encapsulated tumor ; LN, lymph node tissue ; C, cicatricial area with the presence of melanin.
Lower panel : the same cryosection after excision of microdissected areas (1 mm in diameter) used for determination of CTL 16 frequencies among the $CD8^+$. cDNA of each area was tested by clonotypic PCR amplifications of TCR 16 Vα and Vβ rearrangements and by CD8β quantitative PCR (Taqman method). The number of all CTLs in each area was calculated considering a previously defined estimation of the number of CD8β transcripts in one CTL.

Patients vaccinated with dendritic cells pulsed with a single MAGE-3 peptide presented by HLA-A1

Eleven far advanced patients received 5 vaccinations at 14 days intervals. Regressions of individual metastases were evidenced in 6 of them (7). We monitored the anti-vaccine cytolytic T lymphocyte (CTL) response in 3 regressing patients and in 1 progressing patient, resorting to two parallel approaches. The first approach was a MLPC- A1/MAGE-3 tetramer analysis. In a second approach (*ex vivo*-tetramer), blood lymphocytes were assayed immediately with the A1/MAGE-3

tetramer, the labeled cells were cloned and their diversity analyzed by TCR sequencing. A polyclonal CTL response against the MAGE-3.A1 antigen was observed in the 3 patients who showed regression of some of their metastases after vaccination (# 04, 06 and 09) (see Figure 2) . The frequency estimated after vaccination ranged from $4x10^{-6}$ to $1x10^{-3}$ among $CD8^+$ blood lymphocytes, corresponding to an amplification factor of 10 to 500 above the value found before immunization.

Among the various clonotypes found in each patient, two or three of them were found repeatedly and represented about 60% of the CTL response. Such response was not found in the patient who didn't show any regression of his metastases (# 11).

For patient # 04, clonotypic PCR were set up for clonotypes 1 and 5 and used to test cDNAs derived from freshly thawed groups of lymphocytes. Frequencies in line with those obtained by the MLPC-tetramer and *ex vivo*-tetramer approaches were obtained, indicating that our culture conditions allowed the survival and amplification of most of CTL precursors (CTLp)(8).

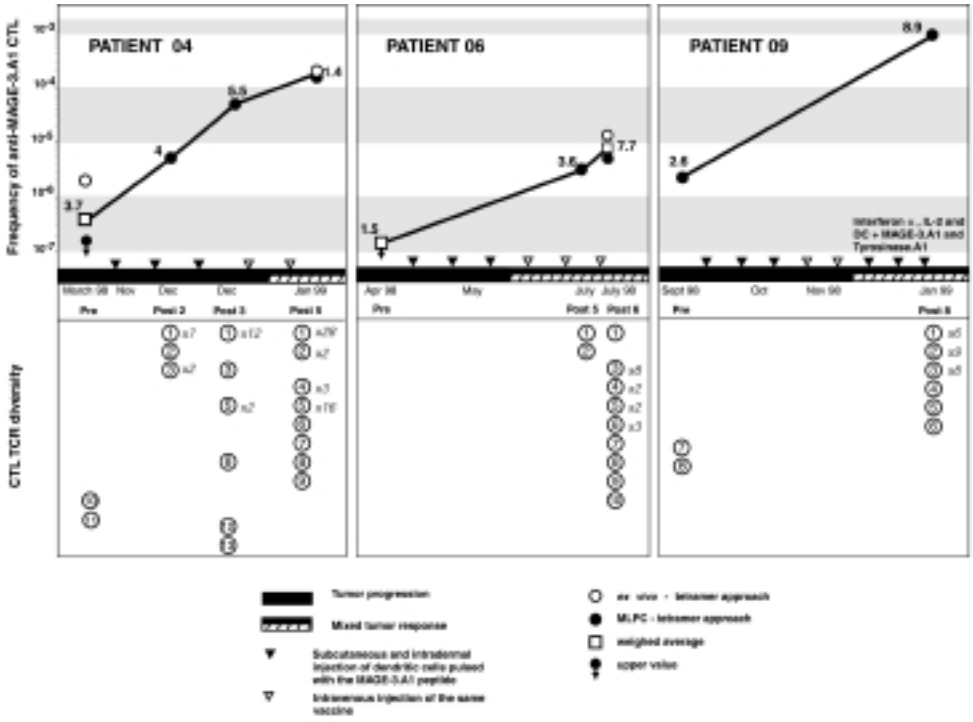

Fig. 2. Frequency and diversity of anti-MAGE-3.A1 CTL.
Bottom panels represent CTL clones, with different numbers for each TCR sequence, and the occurence of repeated clones. TCR numbering starts at 1 for each patient. Similar numbers assigned to CTL of different patients do not represent TCR similarity.

Patients vaccinated with dendritic cells pulsed with multiple peptides.

A new protocol was established by the group of G. Schuler, using dendritic cells pulsed with at least three different tumor epitopes. While HLA-A1 patients received dendritic cells pulsed with the MAGE-1.A1 and the MAGE-3.A1 peptides, HLA-A2 patients were vaccinated with autologous dendritic cells pulsed with the MAGE-4.A2 and the MAGE-10.A2 peptides (9, 10).

We analyzed the T cell response of one of these HLA-A2 patients (A2-R-12), using the MLPC-tetramer approach. As shown in figure 3, no CTLs directed against the MAGE-4.A2 epitope were obtained after the 5 vaccinations. On the contrary, the frequency of anti-MAGE-10.A2 CTLp was evaluated to be $2.35x10^{-5}$ after vaccination. As in the first study with dendritic cells pulsed with the MAGE-3.A1 peptide, the TCR analysis of the CTL clones obtained evidenced a polyclonal response. No CTLs were isolated from the blood sample collected before vaccination, leading to a frequency estimate below $5.3x10^{-7}$ of CD8 T cells. The CTL

responses of other patients included in this protocol are currently being examined to determine if, as suggested for patient A2-R-12, some peptides immunize better than others.

Even though our data provide no information about the effector mechanisms responsible for the observed regressions in these patients, they suggest that DC vaccinations can induce CTL responses that differ from that induced by vaccination with peptide alone in term of their higher frequency and polyclonality.

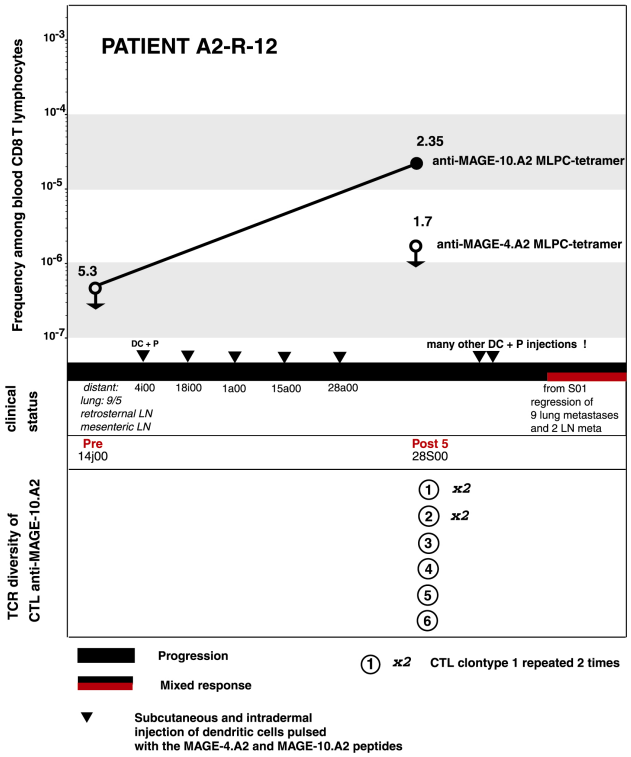

Fig. 3.

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II. CYTOKINE FUNCTION

OVERVIEW

Our interest in cytokines stemmed from the study of various murine cell lines whose in vitro growth was found to be dependent on supernatant from activated T cells. By purifying the active factor from such supernatants, we identified a mouse protein that stimulates the growth of B-cell hybridomas (1). This factor, now designated Interleukin 6 (IL-6), turned out to play a pivotal role as an accessory signal in the early steps of immune responses. IL-6 is also a growth factor for many mouse plasmacytomas and human myelomas, and seems to play a pathogenic role in myeloma patients (2).

Another cytokine, IL-9, was similarly identified at the branch as a growth factor for mouse T Helper clones (3). In vitro, IL-9 exerts activities on T cells, B cells, mast cells, erythroid and myeloid progenitors, as well as immature neurones. However, its major biological activities have been mainly enlightened by the analysis of transgenic mice that overexpress this cytokine.

First, IL-9 transgenic mice, that have a high level of this cytokine in all tissues, are characterized by a high susceptibility to the development of T cell lymphomas. Indeed, approximately 5% of these mice spontaneously develop thymic lymphomas (4). In addition, they show a very high sensitivity to the oncogenic effect of very small doses of chemical mutagens or irradiation. By contrast, normal T cell subsets do not seem to be affected by IL-9 overexpression.

Another major aspect of IL-9 biology is its growth and differentiation activity on mast cells. IL-9 transgenic mice show increased numbers of mast cells in the gut and airways (5). As a result, these animals are particularly resistant to infection by intestinal nematodes such as *Trichinella spiralis* or *Trichuris muris*. The effect of IL-9 on pulmonary mast cells might be related to genetic data pointing to *IL9* as a candidate susceptibility gene for asthma.

Finally, a puzzling activity of IL-9 is a selective increase in the peritoneal B1b cell subpopulation (6). Although the specificity of these cells is far from clear, they might be related to some auto-immune processes.

In line with the oncogenic activity of IL-9 in transgenic mice, this cytokine was shown to be a potent anti-apoptotic factor for T cell lymphomas (7). Interestingly, in the same anti-apoptotic model, we observed that the I-309 chemokine also inhibits some apoptotic processes (8). Further studies have now allowed for the characterization of the I-309 receptor and provide evidence that a distinct activation pathway, namely the Ras-MAP-kinase pathway is responsible for this I-309 activity .

By contrast, the anti-apoptotic effect of IL-9 does not involve MAP-kinases but is mediated by the JAK/STAT pathway. Studies of the mode of action of IL-9 at the molecular level showed that, 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 are associated with IL-9R and γc,

respectively. A single tyrosine residue of the IL-9R is then phosphorylated and acts as a docking site for 3 transcription factors, STAT-1, -3 and -5, which become phosphorylated and migrate to the nucleus, where they activate the transcription of a number of genes. This pathway is common to many cytokines but is often dispensable for their known biological activities. For IL-9, we demonstrated that activation of the STAT transcription factors is crucial for all the known effects of IL-9 (9). STAT3 seems to be the main mediator of IL-9-induced differentiation of T lymphocytes and mast cells, and STAT5 is mainly involved in proliferative responses of B and T lymphocytes. Both STAT3 and STAT5 can confer protection against corticoid-induced apoptosis, however. Finally, activation of the three STAT transcription factors was required for optimal cell growth, suggesting additive or synergistic effects in the regulation of gene expression by these proteins (10).

Therefore, we are now focusing on the characterization of genes whose expression is regulated by IL-9 through the activation of STAT transcription factors.

Two of these genes, *bcl3* and *M-ras*, reflect a secondary layer of signal transduction modulation by IL-9. Bcl-3 is a member of the IκB family and favors the nuclear translocation and DNA binding of p50/p50 homodimers of the NF-κB transcription factors. Upregulation of *bcl3* expression by IL-9 thus represents a new and unexpected mechanism of NF-κB activation by cytokines (11).

M-ras is another example of a signal transduction-related gene regulated by IL-9. This new member of the Ras family is induced by IL-9 in mouse T cells and exerts some transforming activities since it can (i) mediate transformation of NIH-3T3 fibroblasts, (ii) promote cytokine-independent proliferation of lymphocyte cell lines, and (iii) protect T lymphomas against corticoid-induced apoptosis (12).

The search for IL-9-induced genes led us to characterize a new gene encoding a 180 amino acid protein, that shows a weak but significant sequence homology with IL-10. This protein, originally designated IL-TIF for IL-10-related T-cell-derived Inducible Factor, is induced by IL-9 in thymic lymphomas, T cells and mast cells. This new cytokine has now been renamed IL-22 and has become a major research topic in our group (13, 14).

In 2001, the group of Stefan Constantinescu joined the Institute and reinforced and extended our focus on signal transduction mechanisms by cytokine receptors such as the receptors for erythropoietin (EPO) and thrombopoietin (TPO). The main topic of this group is to elucidate the mechanisms by which the different chains of cytokine receptors associate between each other and with JAK kinases, including the orientation of the transmembrane and cytosolic domains, and the consequences of these interactions for receptor traffic and signalling.

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CYTOKINES IN IMMUNITY AND INFLAMMATION

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Monique STEVENS, Technician
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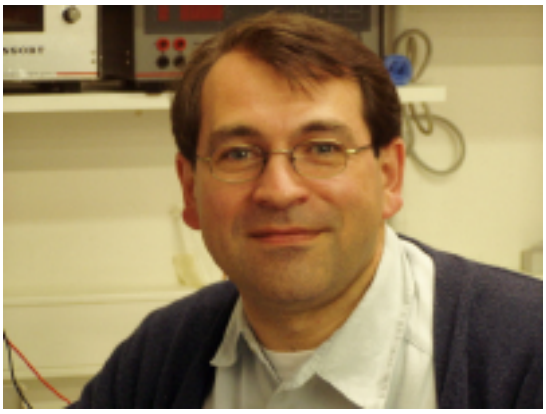

The cytokine group studies the biology of Interleukin-9 (IL-9) and IL-22, two cytokines discovered at the Branch. IL-9 is a TH2 cytokine that plays a role in immune responses against intestinal parasites and asthma. IL-22, originally identified as a gene induced by IL-9 in T lymphocytes, upregulates the production of acute phase reagents in the liver. Its activity in inflammatory responses is modulated by a specific antagonist, the IL-22 binding protein (IL-22BP). The role of IL-9 and IL-22 in inflammation is currently being investigated using transgenic and gene-targeted mice for these cytokines and their receptors.

Interleukin 9

Jean-Christophe Renauld, Jamila Louahed, David Darcaigne, Laurent Knoops, Valérie Steenwinckel, Brigitte de Lestré, Monique Stevens, Emiel Van Roost

Interleukin-9 (IL-9) was discovered in our group in 1989, through its ability to sustain antigen-independent growth of certain murine T helper clones. We further identified human IL-9 by cross-hybridization with the mouse gene. Although IL-9 did not turn out to be a T cell growth factor for freshly isolated T cells, it was found particularly potent on T cell lymphomas, as an anti-apoptotic agent. To determine the biological activities of this factor, we generated transgenic mice overexpressing this cytokine. Analysis of these animals disclosed three essential properties of IL-9: its tumorigenic potential in T lymphocytes, its stimulatory activity on a particular subset of B lymphocytes, and its activity on mast cells and eosinophils with consecutive implications in asthma.

IL-9-transgenic mice : T cell lymphomas

IL-9 transgenic animals showed normal T cell development and T cell numbers but spontaneously developed thymic lymphomas at low frequency (5%) when maintained in a conventional environment. Two lines of evidence indicate that IL-9 is not a conventional oncogene but rather favors tumor development in response to exogenous stimuli. First, the tumor incidence was significantly lower when mice were maintained under pathogen-free conditions. Secondly, all IL-9 transgenic mice developed T cell lymphomas when exposed to subliminal doses of a chemical carcinogen or to irradiation, that were innocuous in wild type mice (1). The above mentioned anti-apoptotic activity of IL-9 provides an attractive explanation for these observations, namely that IL-9 could lead to increased survival of abnormal cells generated by exposure to minimal doses of oncogenic stimuli. The potential implication of IL-9 in oncology was also confirmed in human systems by its constitutive expression in Hodgkin lymphomas.

IL-9-transgenic mice : B1 cell expansion

Further analysis of these IL-9-transgenic mice showed that a particular B lymphocyte population, called B-1 lymphocytes and usually restricted to the peritoneal and pleuropericardial cavities, were dramatically expanded in response to IL-9 overproduction. In addition, such cells were also found in the blood of IL-9 transgenic mice. This observation is reminiscent of mice that are prone to the development of diseases that are characterized by the production of autoantibodies, such as Systemic Lupus Erythematosus, and suggests that

IL-9 might play a role in some autoimmune processes (2).

IL-9-transgenic mice : parasite infections and asthma

In addition, IL-9 transgenic mice were found to harbor increased numbers of mast cells in the intestinal and respiratory epithelia, and were also characterized by a general hypereosinophilia. This phenotypic characteristic was found to increase the capacity of these animals to expel nematodes like Trichinella spiralis or Trichuris muris., suggesting that IL-9 administration could protect susceptible hosts against these parasites. This was confirmed by taking advantage of a new strategy of anti-cytokine vaccination: mice vaccinated against their own IL-9 failed to expel T.muris parasites and had a decreased eosinophilic response against the parasite (3).

The other side of the coin was the discovery that IL-9 overexpression such as that characterizing the IL-9 transgenic animals resulted in bronchial hyperresponsiveness upon exposure to various allergens. The potential aggravating role of IL-9 in asthma was confirmed by genetic analyses performed by others and pointing to both IL-9 and the IL-9 receptor genes as major candidate genes for human asthma.

IL-9 receptor and signal transduction

Jean-Christophe Renauld, Laurent Knoops, Diane Lejeune, Monique Stevens, Emiel Van Roost

Analysis of the mode of action of IL-9 at the molecular level was initiated in 1992 by the cloning of the murine and human IL-9 receptor (IL-9R) cDNAs (4). By further dissecting the signal transduction cascade triggered by IL-9, we showed that, 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 are associated with IL-9R and γc, respectively. A single tyrosine residue of the IL-9R is then phosphorylated and acts as a docking site for 3 transcription factors of the STAT family, STAT-1, -3 and -5, which become phosphorylated and migrate to the nucleus, where they activate the transcription of a number of genes. This pathway is common to many cytokines but is often dispensable for their biological activities. For IL-9, our group demonstrated that activation of the STAT transcription factors is crucial for all the effects of IL-9 studied on various cell lines, including positive and negative regulation of cell proliferation, as well as inhibition of corticoid-induced apoptosis in T cell lymphomas. Further analysis demonstrated that STAT-1, -3 and -5 play specific, redundant and synergistic roles in the different activities of IL-9 in vitro (5).

The pathways responsible for IL-9-induced proliferation were studied in details, and this process was found to depend mainly on the activation of STAT-5, on the recruitment of the IRS-1 adaptor, and on the activation of the Erk MAP-Kinase pathway.

The signal transduction pathway downstream the IL-9 receptor is illustrated in Fig. 1.

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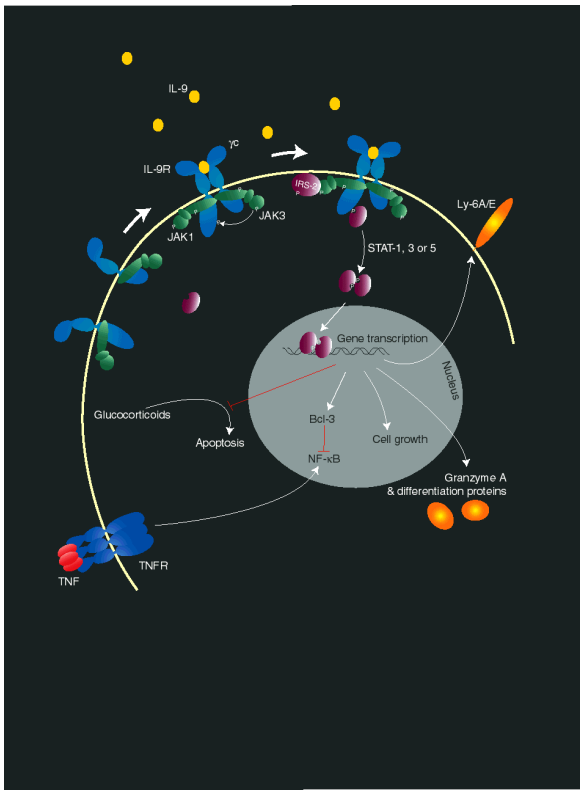

Fig. 1

Anti-apoptotic activity of I-309receptor and signal transduction

Jean-Christophe Renauld, Jacques Van Snick, Jamila Louahed

Incidentally, our studies of this particular model of the regulation of cell death by cytokines, lead them to purify another protein called I-309, originally described as a human chemotactic factor, and that turned out to exert a significant anti-apoptotic activity for thymic lymphomas (6). However, I-309 and IL-9 trigger completely different pathways and it was shown that the I-309

anti-apoptotic activity was dependent on the activation of G-proteins and the Ras/MAPKinase pathway, whereas the IL-9-mediated effect was not. More recently, we showed that a viral protein related to human chemotactic factors (vMIP-I), and isolated from Herpes viruses that induce T cell tumors, has the same anti-apoptotic activity by binding to the I-309 receptor

IL-9-induced genes

Jean-Christophe Renauld, Jacques Van Snick, Laure Dumoutier, Jamila Louahed, Laurent Knoops, Diane Lejeune, Monique Stevens, Emiel Van Roost

To further characterize the mechanisms involved in the anti-apoptotic activity of IL-9 in this experimental model, we sought to identify genes induced by IL-9 in T cell lymphomas. Among the genes we identified, three open unexpected perspectives: BCL3, M-Ras and IL-TIF/IL-22.

BCL3 : indirect modulation of NF-κB

BCL3 is a gene originally identified at the breakpoint of translocations found in B cell leukemia, resulting in its transcriptional activation. The BCL3 protein interacts with NF-κB transcription factors and its induction by IL-9 represents a novel mechanism of NF-κB regulation by cytokines, and a new crosstalk between the JAK/STAT and NF-κB signal transduction pathway (7). BCL3 induction might play a role in the antiapoptotic activity of cytokines such as IL-4 and IL-9.

M-Ras : transcriptional regulation of the Ras-MAPKinase pathway

M-Ras is a new member of the Ras oncogene superfamily. The Ras proteins are known to regulate various cellular processes such as proliferation and apoptosis, when they are in their activated form, in association with a GTP molecule. Contrasting with the potent upregulation of M-Ras expression, M-Ras was not activated by IL-9 at the level of GTP binding. However, other cytokines such as IL-3 increased GTP binding to M-Ras, suggesting that M-Ras induction might represent a new mechanism of cooperativity between cytokines. Constitutively activated M-Ras mutants trigger the MAP Kinase pathway and induce proliferation of cytokine-dependent cells (8).

IL-TIF/IL-22 : a new cytokine structurally related to IL-10

IL-TIF is a new gene that turned out to encode a 179 amino acid long protein, including a potential signal peptide, and showing a weak but significant sequence homology with IL-10. This protein, originally designated IL-TIF for IL-10-related T-cell derived Inducible Factor, was later renamed IL-22. Its expression is induced by IL-9 in thymic lymphomas, T cells and mast cells and by lectins in freshly isolated spleen cells. In addition, constitutive expression of IL-22 was detected by RT-PCR in thymus and brain, suggesting that the role of this new factor is not restricted to the immune system. Preliminary experiments showed that IL-22 induces STAT activation in various cell lines, suggesting that this factor might mediate some of the activities of IL-9. Biological activities of IL- 22 include the induction of acute phase proteins in liver (9). Recombinant human IL-22 was produced (with D. Colau, LICR) and its crystallograhic structure solved. Despite its structural homology with IL-10, IL-22 fails to recapitulate any of IL-10 biological activities.

Analysis of the genome databases leads to the identification of a new receptor belonging to the IL-10 receptor family (10). This gene is located in the chromosome 6q24, at 24 kb from the IFNGR1 gene and at 152 kb from the IL-20R. It encodes a protein of 231 amino acid, showing 33 % and 34 % amino acid identity with the extracellular domains of the IL-22R and the IL-20R, respectively, but no cytoplasmic nor transmembrane domains were found. IL-22BP is highly expressed in the placenta, in the breast, in the mammary gland and in the skin. A specific interaction was demonstrated between insolubilized IL-22 and an IL-22BP-Ig fusion protein. Moreover, recombinant IL-22BP could block IL-22 biological activity demonstrating that this protein can act as an IL-22 antagonist.

Although IL-22 does not share any biological activity with IL-10, these 2 cytokines share a common component of their respective receptor complex, IL-10Rß. Anti-IL-10Rß antibodies indeed block the IL-22-induced acute phase response in HepG2 cells (9). All receptor complexes for IL-10-related cytokines include a long chain and a short chain, based on the length of the cytoplasmic domain of these transmembrane proteins. IL-10Rß is a typical short chain component, with only 76 amino acids in the cytoplasmic domain, whose main function seems to consist in recruiting the Tyk2 tyrosine kinase. In addition to IL-10R ß, IL-22 signalling requires the expression of a long chain protein, called IL-22R and comprising a 319 amino acid long cytoplasmic domain. This chain associates with Jak1, and is responsible for the activation of cytoplasmic signalling cascades such as the JAK/STAT, ERK, JNK and p38 MAP kinase pathways.

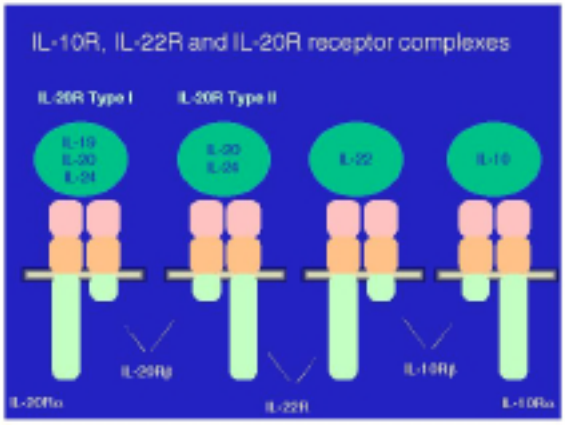

Fig. 2

In addition to its role in IL-22 binding and signalling, the IL-22R chain also forms a functional heterodimeric receptor complex by associating with IL-20R ß, the second short chain member of the IL-10R-related receptor family. This complex mediates STAT-1 and –3 activation by IL-20 and IL-24, but not by IL-22 (11). In addition, IL-20 and IL-24 can also bind to other complexes consisting of IL-20Rα and IL-20Rß. This promiscuity in cytokine receptor usage is illustrated in Fig 2.

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STRUCTURE AND FUNCTION OF CYTOKINE RECEPTORS

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Cytokines and their receptors are critical for formation of mature blood cells and for the function of the immune system. Signaling by cytokine receptors is triggered by ligand-induced changes in receptor dimerization/oligomerization, which induces the activation of cytosolic Janus tyrosine kinases (JAK). Activated JAK proteins phosphorylate downstream proteins, like receptors themselves, signal transducers and activators of transcription (STAT) proteins and a variety of other signaling proteins. Phosphorylated STAT proteins dimerize in the cytosol and are translocated to the nucleus where they bind to specific promoter sequences and regulate transcription. We study the signal transduction mechanisms and biologic functions of cytokine receptors such as the receptors for erythropoietin (Epo), thrombopoietin (Tpo), interleukin 2 (IL2) and interleukin 9 (IL9). The assembly of cell-surface receptor complexes, the structure and orientation of the transmembrane (TM) and cytosolic juxtamembrane (JM) domains, and the regulation by JAK kinases of receptor traffic are major focuses. We also study the mechanisms by which STAT proteins become constitutively activated and how they function in transformed hematopoietic or patient-derived leukemia cells.

Determination of the interface and orientation of the activated erythropoietin receptor dimer

Nadine Seubert, Yohan Royer, Katharina Kubatzky, Nicole El-Najjar

Epo binding to the erythropoietin receptor (EpoR) results in survival, proliferation and differentiation of erythroid progenitors into mature red blood cells (1). We have shown that, in the absence of Epo, the cell-surface EpoR is dimerized in an inactive conformation which is stabilized by interactions between the TM sequences (2). Epo binding to the extracellular EpoR domain induces a conformational change of the receptor which results in the activation of cytosolic JAK2 proteins. The α-helical orientation of the (TM) and cytosolic JM domains is crucial for receptor activation (3). Specifically, in collaboration with Lily Huang and Harvey Lodish, Whitehead Institute, Cambridge, MA, USA, we have identified a number of key residues in the EpoR cytosolic JM domain which are required for switching on the activity of JAK2 and initiate signaling (4).

To identify the residues that form the interface between the receptor monomers in the activated EpoR dimer we have replaced the EpoR extracellular domain with a coiled-coil dimer of α-helices. Because coiled-coils have a characteristic heptad repeat with hydrophobic residues at positions a (1), d (4), and a (7), the register of the coiled-coil α-helices is imposed on the downstream TM α-helix and intracellular domain. This allows the prediction of the position (on an α-helix) of the residues that will be in the interface of the activated EpoR dimer. We have generated seven different constructs where all seven possible orientations were imposed by the coiled-coil on the fused TM and intracellular domain of the EpoR. All seven fusion proteins are expressed when transduced in

cytokine-dependent cell lines and reach the cell-surface. However, only two of the seven fusion proteins showed activity represented by stimulation of proliferation of cytokine-dependent cell lines and erythroid differentiation of primary fetal liver cells. The predicted dimeric interfaces of the two active fusion proteins are very close, emphasizing the notion that a unique dimeric EpoR conformation is required for activation of signaling. At present we are characterizing the signaling events induced by the two active coiled-coil-EpoR fusion proteins and we are performing Cys scanning of the EpoR TM domain and cross-linking assays in order to prove that the dimeric active interface we have identified can be demonstrated in the wild type receptor dimer

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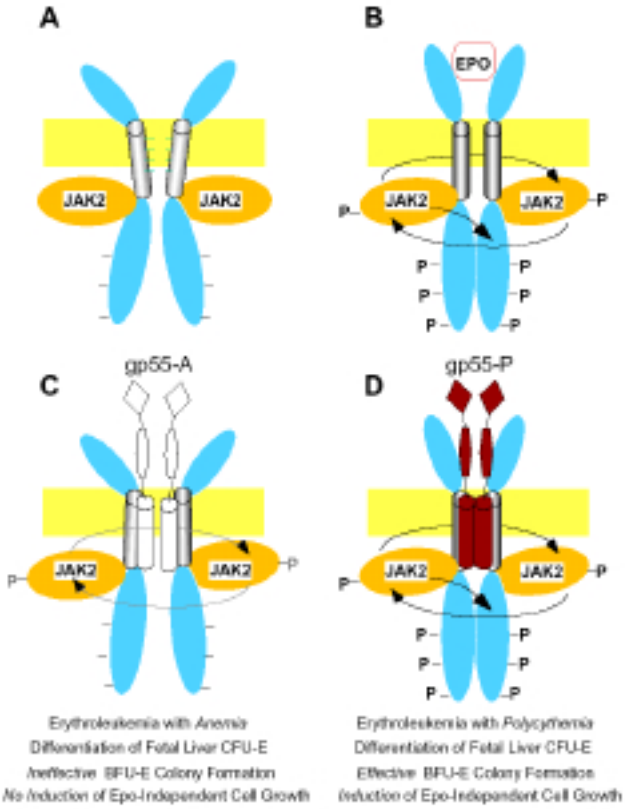

(A) The erythropoietin receptor (EpoR) is an inactive dimer on the cell-surface in the absence of ligand due to interactions between the transmembrane (TM) domains (interrupted line). Cytosolic Janus kinase 2 (JAK2) is bound to the receptor juxtamembrane domain and stimulates receptor folding and traffic to the cell-surface.

(B) Erythropoietin (Epo) binding induces a conformational change in the extracellular domain which is transmitted via the α-helical TM domain to receptor residues in the juxtamembrane domain that contact JAK2 and switch its activity on. Activated JAK2 phosphorylates (-P) itself, the receptor cytosolic domain and many other signaling proteins leading to survival, proliferation and differentiation of erythroid progenitors into mature red blood cells.

(C) Co-expression of the EpoR with the gp55-A envelope protein of the Spleen Focus Forming Virus results in cell-surface complex formation due to specific interactions between the TM domains. The EpoR transmembrane dimer, which maintains the EpoR inactive, is disrupted by the interaction with gp55-A. The receptor acquires a conformation, which allows weak constitutive activation resulting in erythroleukemia with low numbers of red blood cells *(anemia)*.

(D) Co-expression of the EpoR with the viral gp55-P envelope protein results in cell-surface complex formation due to specific interactions between the TM domains. In this complex the EpoR acquires a dimeric conformation very similar to that induced by Epo, which results in strong constitutive activation of EpoR signaling leading to erythroleukemia and massive production of mature red blood cells *(polycythemia)*.

Structural studies on the transmembrane and juxtamembrane cytosolic sequences of the EpoR

Katharina Kubatzky, Nicole El-Najjar

The structure of the transmembrane and cytosolic domains of cytokine receptors remains a mystery. Due to our previous work showing that the junction between the EpoR TM and intracellular domain is rigid (3) we hypothesized that the important structured segments of the cytosolic domain must contain the cytosolic JM segment. We have cloned the cDNAs coding for the seven coiled-coil-EpoR fusion proteins in the pET31b vector in order to produce recombinant fusion proteins in quantities amenable for biophysical and structural studies. In collaboration with the group of Steven Smith, SUNY, Stony Brook, NY, USA, we will determine the NMR structure of the EpoR TM and cytosolic JM domains in the active and inactive coiled-coil-EpoR fusion proteins. The structural studies will be facilitated by the already known structure of the coiled-coil part of the fusion proteins. Comparing the structure and biophysical properties of the active and inactive coiled-coil-EpoR fusion proteins we aim to determine the conformational requirements of the activated state of the receptor. The information obtained by studying the model coiled-coil-EpoR fusion proteins will be then tested on the wild type EpoR. In addition, the recombinant form of the two active coiled-coil-EpoR fusion proteins will be used as baits in order to identify receptor binding proteins in cellular lysates.

Traffic of cytokine receptors to the cell surface

Yohan Royer

Traffic and cell-surface expression of the EpoR critically depend on the ability of EpoR to bind JAK2 intracellularly (4). In the absence of JAK2 the receptor does not get transported from the endoplasmic reticulum (ER) to the Golgi aparatus and does not acquire EndoH resistance. Strikingly, chimeric proteins that contain the EpoR intracellular domain and the extracellular domains of IL9Rα, IL2Rβ or of the common γ chain, are expressed on the cell-surface as a function of coexpressed JAK2. Moreover, we have observed that in hematopoietic cells overexpressing JAK proteins several cytokine receptors are expressed at significant higher levels on the cell-surface. Particularly, the IL9Rα which requires JAK1 for signaling is expressed at higher levels on the cell-surface when JAK1 but not JAK2 or JAK3 is overexpressed. Our working hypothesis is that the N-terminus FERM domain of JAK proteins exerts a generic pro-folding effect on cytosolic domains of cytokine receptors. We are testing this hypothesis on several different cytokine receptors and are investigating the link between proper folding in the ER and transport to the cell-surface.

Structure and function of the extracellular domain of the EpoR (Katharina Kubatzky, Yohan Royer). Recombinant Epo is the leading drug of modern biotechnology and is widely used in the clinic for treatment of anemias of different causes, such as chronic renal failure, anemia induced by cancer chemotherapeutic agents, anemia of AIDS patients receiving AZT, or anemias of prematurity, rheumatoid arthritis and myelodisplasia. Apart from being expressed in erythroid progenitors, megakaryocytes and endothelial cells, the EpoR is expressed in neurons where it can protect from apoptosis induced by hypoxia. The present challenge is to design a small molecule mimetic or partial mimetic of Epo which should be non-toxic and non-immunogenic. The mechanism by which Epo binding to the extracellular domain changes the conformation of the EpoR is not known but is relevant for the efforts to isolate small molecule activators of EpoR. Epo binds to three loops on each monomer of the EpoR extracellular domain (EpoR-ECD). The same loops are involved in binding of a mimetic peptide and in contacts between un-liganded extracellular domains. Using insect cell produced EpoR-ECD we have shown that Cys181 of the ECD can be used to cross-link two ECD monomers in the presence of ligand. Using mutagenesis and recombinant production of the EpoR-ECD in insect cells we will investigate the conformation of the EpoR-ECD in the presence and absence of ligand as well as the conformation of a mutant constitutively active form of the EpoR-ECD (EpoR R129C) .

Signaling by the thrombopoietin receptor

Judith Staerk

The thrombopoietin receptor (TpoR) is essential for formation of platelets, for renewing hematopoietic stem cells and for expanding myeloid progenitors (5). Like the EpoR, the TpoR is thought to signal by activation of JAK2, of several STATs (STAT1, 3 and 5) as well as of MAP-kinase, PI-3-kinase and AktB. However, TpoR and EpoR signal quite differently since only TpoR can induce hematopoietic differentiation of embryonic stem cells or stimulate the earliest stages of hematopoiesis in immature hematopoietic cells. In contrast, only EpoR can support efficient formation of mature red cells. Since both EpoR and TpoR are members of the homodimeric class of cytokine

receptors we have started to compare signaling and gene expression induced by these two receptors as well as the orientation of their intracellular domains in the activated state. We have already identified a major difference between TpoR and the EpoR, namely that several orientations of the TpoR are compatible with inducing cell proliferation but only a restricted dimeric conformation is compatible with the induction of intercellular adhesion. By constructing chimeric receptors we attempt to identify the relevant sequences which confer to the TpoR and EpoR their specific biologic activities in immature hematopoietic progenitors and committed erythroid progenitors, respectively

Signaling by the receptors for IL2 and IL9 via the common γ chain (γ-c)

Yohan Royer

γ-c is a cytokine receptor that is shared by the receptor complexes of several cytokines, such as IL2, IL4, IL7, IL9 and IL15. γ-c binds and activates JAK3. Humans that lack the γ-c or have mutations in JAK3 develop severe combined immunodeficiency. We are investigating the assembly of IL9Rα and IL2Rβ with the common γ chain. We have employed PCR-directed mutagenesis to test the involvement of a conserved cytokine receptor JM hydrophobic motif (3) in IL9Rα and IL2Rβ signaling. It appears that IL9Rα has quite different sequence requirements than IL2Rβ for signaling, although both utilize γ-c. While IL9Rα is rather similar to the EpoR, IL2Rβ does not require hydrophobic residues at positions –1, -2 and –6 from Box 1. We are also investigating the sequence requirements of γ-c JM domain for activating JAK3 and interacting with JAK1. In collaboration with Jean-Christophe Renauld we attempt to isolate novel IL9Rα and γ-c mutants that would reveal the precise domains of IL9Rα and γ-c that trigger activation of JAK1 and JAK3.

Sequence–specific interactions between transmembrane domains

Nicole El-Najjar

Two transmembrane viral enevelope proteins (gp55-P and gp55-A) belonging to the polycythemic (P) and anemic (A) Spleen Focus Forming Viruses (SFFV) strains, can activate the EpoR when co-expressed in the same cell (6). In collaboration with Yoav Henis, Tel-Aviv University, Israel, we have shown that both the gp55-A and gp55-P TM domains specifically interact with the TM domain of the EpoR. gp55-A weakly activates the receptor leading to erythroleukemia with low number of red blood cells (*anemia*). However, gp55-P fully activates the EpoR to stimulate proliferation and differentiation of erythroid progenitors leading to both erythroleukemia and massive red blood cell production (*polycythemia*). The activation of EpoR by gp55-P results from a highly specific interaction between the membrane spanning sequences of the two proteins: Ser238 of the murine EpoR TM and Met390 of the gp55-P TM are critical determinants of this interaction (7). Taking advantage of this specific interaction we are constructing a genetic system where the TM sequence of gp55-P is randomized or replaced with short sequences derived from a lymphoid cDNA library in order to select for novel sequences capable of functioning as TM domains (transmembrane domain trap), of binding EpoR and of activating the EpoR. Several genetic approaches exist to probe the ability of a particular TM sequence to homodimerize/homo-oligomerize but no assay has been reported for selection of hetero-interactions between TM sequences. In our system activation of EpoR signaling will result in cell survival and proliferation which represent a powerful selection.

Constitutive activation of JAK-STAT signaling pathways and genes targeted by STAT5 in transformed hematopoietic and patient-derived leukemia cells

Virginie Moucadel, Yohan Royer, Judith Staerk

Cytokine stimulation of cytokine receptors, induces transient activation of the JAK-STAT pathway. In contrast several mutant forms of cytokine receptors (i.e. EpoR R129C or TpoR S498N) have been isolated that signal constitutively (reviewed in (1, 8, 9)). Such receptors are permanently dimerized in an activated state and induce the biologic effects of the wild type receptors as well as leukemic cell transformation. In cultured cells this process is studied by expressing oncogenic forms of cytokine receptors in cytokine-dependent cells and assaying for their transformation into cells that grow autonomously, in the absence of any cytokine. In the transformed cells many of the transient signaling events induced by cytokines are detectable permanently, i.e. ligand-independent phosphorylation of JAK and STAT proteins or high levels of nuclear activated STATs especially STAT5 and STAT3. A similar picture has been noted in patient derived leukemia cells, where constitutively active STAT proteins have been reported in a majority of myeloid and lymphoid leukemia patients. The critical questions we would like to answer concern the mechanisms by which the JAK-STAT remain permanently activated in transformed cells and which genes are regulated by constitutively active STAT proteins in leukemic cells.

First, by using bicistronic retroviral vectors and cell sorting we have generated hematopoietic cells that express 5-10 fold higher levels of JAK proteins (such as JAK1, JAK2 and JAK3) or STAT5. Pools of cells that overexpress JAK or STAT proteins but remained cytokine-dependent for growth and survival were isolated. Like the parental hematopoietic cells these JAK/STAT overexpressing cells can be transformed to growth-factor independence by expression of constitutively active forms of cytokine receptors. Because the levels of expression of JAK and STAT proteins in these cells are amenable to detailed biochemical analysis we are using immunoprecipitation of the overexpressed JAK-STAT proteins and mass spectrometry (in collaboration with the laboratory of Mark Rider) in order to map novel phosphorylation sites and to isolate and identify associated proteins.

In a second approach we are attempting to identify the promoters actually bound by STAT proteins in living cells in physiologic and pathologic situations. We use a modified version of the chromatin immunoprecipitation assay pioneered by Alex Varshavsky in conjunction with DNA microarray gene profiling. Control, ligand-activated or transformed cells are treated with 1% formaldehyde to cross-link genomic DNA with protein. Cells are lysed, chromatin is sonicated to a certain average length (i.e. 600 bp) and then purified. The isolated DNA- protein complexes are immunoprecipitated with antibodies directed against STAT proteins or with control antibodies. After elution and reversal of cross- linking genomic DNA will be linked to adapters and PCR amplified and sequenced. The isolated genomic fragments will be screened for the presence of STAT-binding sites and tested for the ability to regulate transcription of reporter genes. Newly identified genes regulated by such genomic sequences will be expressed in bicistronic retroviral vectors that allow wide expression of cDNAs at physiologic levels (10). In preliminary experiments we have shown that using chromatin immunoprecipitation and PCR amplification we could isolated several genomic sequences that were bound by STAT5 after ligand addition.

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