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Research Report – 2004

DEPARTMENT OF EXPERIMENTAL ONCOLOGY
Head: Associate Professor Adam Opolski, Ph.D.

Laboratory of Experimental Anticancer Therapy
Head: Associate Professor Adam Opolski, Ph.D.
Studies on the mechanisms of tumor progression and metastasis and on the effects of experimental antitumor therapy

The effect of Mg deficiency on transplantable mouse tumor growth and metastasis

The results obtained indicate a significant retardation of primary tumor growth (up to 70%) in mice receiving a Mg-deficient diet. However, Mg repletion caused a significant increase in primary tumor burden in these mice. Analysis of the cell cycle distribution showed a reduced percentage of cells in the S phase and increased G_0/G_1 phase of the cell cycle in LLC tumors caused by Mg deficiency. This is in agreement with the effect of low Mg level on cell growth observed *in vitro*. Interestingly, in mice inoculated with LLC cells and receiving a low-magnesium diet, a higher metastatic potential, expressed as the number of animals with lung metastatic colonies, was observed compared with control mice. These results demonstrate a direct role of magnesium in tumor growth and also indicate a deleterious effect of low magnesium status on tumor metastasis.

Moreover, we showed that 3 weeks of Mg deficiency in mice resulted in inflammatory processes in lungs, including interstitial and perivascular pneumonia, manifested by the infiltration of leukocytes, plasmocytes, and histiocytes as well as disseminated intravascular coagulation (DIC). These phenomena were accompanied by changes in gene expression assessed by cDNA array. In our studies we identified 26 genes significantly changed by Mg deficiency, mostly involved in anti-oxidative response, regulation of cell cycle and growth, apoptosis, and cell-cell and cell-matrix interactions. We conclude that these changes are related to inflammatory and oxidative processes and the consecutive remodeling occurring in the tissues as a result of Mg deficiency.

Laboratory of Biomedicinal Chemistry
Head: Associate Professor Janusz Boratyński, Ph.D., Eng.

Methotrexate – fibrinogen and glycated fibrinogen antileukemia activity studies

The research at this laboratory focused on the synthesis of non-cross-linked conjugates with reduced hydrophobicity. Owing to new approaches in their synthesis, it became possible to obtain low-toxic conjugates having up to {sixty} drug moieties bound to a single carrier molecule. Some preparations were able to completely cure almost two thirds of mice in the P388 experimental therapy test. The procedure of synthesis of the conjugates is the subject of national and international patent applications. (P363422; PCT PL 0300136)

Physiochemical studies of bacteriophages

A laboratory procedure for the preparation of endotoxin-free bacteriophages of Gram-negative bacteria was developed. The purified bacteriophage preparations meet the requirements for parenteral administration with respect to endotoxin content. The procedure of purification is the subject of a patent application.

DEPARTMENT OF EXPERIMENTAL THERAPY
Head: Professor Michał Zimecki, Ph.D.

Laboratory of Immunobiology
Head: Professor Michał Zimecki, Ph.D.
Studies on the mechanism of action of synthetic and natural immunomodulators of potential application in prevention and therapy

We continued studies on the immunotropic activity of lactoferrin (LF), a milk-derived protein. Specifically, we investigated the ability of LF to reconstitute the immune function in immunocompromised mice, the protective mechanism of LF action in experimental bacteremia, and the adjuvant action of the complex LF-monophosphoryl lipid A (MPL). We showed that in methotrexate (MTX)-treated mice, LF given in drinking water reconstituted the cellular immune response to ovalbumin (OVA) and the secondary, but not the primary, humoral immune response to sheep erythrocytes. The results suggest that LF may exert antiapoptotic action on the activity of memory T cells. Other studies revealed that a strong mobilization of myelopoiesis may explain the protective action of LF in experimental bacteremia when LF was administered intravenously 24 hours before infection with *E. coli*. That effect may be, in part, associated with induction of interleukin 1 (IL-1) and IL-6 production. Significant reduction in the serum level of tumor necrosis factor alpha (TNF-

alpha) upon pretreatment of mice with LF could also contribute to the protective effect of LF in bacteremia.

Studying the interaction of LF molecule with the cells of the immune system *in vivo*, measured by the induction of myelopoiesis or inhibition of lipopolysaccharide-induced TNF-alpha production, showed that preincubation of LF with heparin abolished the effects of LF. Such results indicate that LF interacts with cell receptors via the protein domain rich in basic aminoacids (arginine). Our previous studies showed, on the other hand, that the adjuvant property of LF was mediated by another type of cell receptor recognizing glycans in the LF molecule.

In a search for effective and low-pyrogenic adjuvants, the studies on the LF-MPL complex were continued. We demonstrated that the complex amplified the cellular immune response to OVA compared with LF or MPL applied separately. The method of preparation and potential application of this novel type of adjuvant was patented.

We also studied (in a cooperation with the Institute of Chemistry, University of Wroclaw) the immunosuppressory properties of peptides corresponding to the sequence of beta chain (164-172) of HLA class II molecule. Such peptides might find application in the inhibition of graft rejection. We showed that dimerization of the immunosuppressory fragment of HLA-DR molecule increased its activity, which may be explained by a microaggregation of cell receptors, initiated by dimeric antagonists, thus preventing access of agonists to the receptors.

Laboratory of Immunopathology
Head: Professor Irena Frydecka, M.D.
The mechanisms of immune deficiency in neoplastic diseases

The accumulation of leukemic cells, arrested in G0/G1 phase, and inhibition of apoptosis are well-known features of B-CLL. Because the inhibitory CTLA-4 molecule may be involved in the cell cycle machinery, we have extended our previous study concerning G1 cell cycle phase regulators in B-CLL cells in the context of their association with CTLA-4 expression. We found significantly higher surface and cytoplasmic expression of CTLA-4 in CD19+/CD5+ leukemic cells compared with a control population. Elevated CTLA-4 expression in leukemic cells is associated, at least in part, with CTLA 4 gene polymorphisms. We confirmed our previous observation of increased expression of the G1 phase cell cycle regulators p27KIP1 and the cyclins D2 and D3 in neoplastic B cells. Additionally, we found that CTLA-4 expression positively correlated with cyclin D2 and negatively with cyclin D3, which suggested the possibility of CTLA-4 involvement in the prolongation of the early G1

phase in B-CLL cells. Furthermore, we observed that the proportion of CD19+/CD5+/CTLA-4+ cells positively correlated with the percentage of CD19+ cells in G0/G1 phase and with B CLL progression. Our study provided evidence of CTLA-4 overexpression in B CLL cells and suggests the existence of relationships between this molecule and G1 phase regulators in leukemic B cells.

Experimental studies have demonstrated that CD40/CD154 and CD28/CD152 interactions with their specific B.7 ligands play an important role in the pathogenesis of acute and chronic kidney allograft rejection. The aim of this study was to evaluate the expression of CD154, CD28, and both the surface (s) and intracellular (i) expression of CD152 on freshly drawn, anti-CD3+rIL-2-stimulated peripheral blood CD4+ T cells from kidney transplant recipients, compared with healthy controls. The mean proportion of freshly isolated CD3+/CD4+ cells expressing CD152 and CD154 in all groups of graft recipients was higher compared with controls. The recipients with stable graft function (SGF) were characterized by the highest percentage of CD3+/CD4+/iCD152+ cells before stimulation and the maintenance of a stable percentage of CD3+/CD4+/sCD152+ cells after stimulation with the highest molecular density, accompanied by the lowest percentage of unstimulated CD4+ T cells expressing CD154. In contrast, patients with chronic allograft nephropathy (CAN) exhibited lower frequencies of both freshly isolated CD3+/CD4+/iCD152+ cells and *ex vivo* stimulated CD3+/CD4+/sCD152+ cells, with the highest proportion of unstimulated CD4+ T cells co-expressing CD154. Differences between recipients with and without acute graft rejection were due to a higher percentage of unstimulated CD3+/CD4+/iCD152+ cells in recipients with uneventful early post-transplant course. In conclusion, the results provided the first evidence of a relationship between the pattern of the expression of costimulatory and inhibitory molecules in peripheral blood (PB) CD4+ T cells and clinical outcome of renal transplantation.

CTLA-4, expressed by activated T cells, transduces an inhibitory signal. Patients with relapsing-remitting (RR) and secondary progressive (SP) forms of multiple sclerosis (MS) and healthy subjects were examined for surface/membrane (m) and intracellular (i) expression of down-regulatory CTLA-4 molecule on peripheral blood CD4+ cells. In both groups of MS patients the expressions of mCTLA-4 and iCTLA-4 on freshly isolated CD4+ cells were significantly higher compared with controls. Moreover, the RRMS patients exhibited significantly higher expression of CTLA-4 compared with the SPMS group. After *ex vivo* stimulation there was a significant increase in the expression of mCTLA-4 and iCTLA-4 in the control group. In contrast, in RRMS patients a significant decrease in mCTLA-4

expression was observed, while the iCTLA-4 level was unchanged. In SPMS patients, stimulation did not affect either the surface or intracellular expression of CTLA-4. A positive correlation was found between the rate of MS progression and mCTLA-4 expression on freshly drawn CD4+ cells. We observed no relationship between the A(49)G exon 1 polymorphism and both mCTLA-4 and iCTLA-4 expression in CD4+ lymphocytes from MS patients and controls. Additionally, elevated serum soluble CTLA-4 (sCTLA-4) concentration in patients with RRMS were shown. Up-regulation of mCTLA-4 and iCTLA-4 molecule on freshly isolated CD4+ cells in both MS groups suggests that lymphocytes are continuously activated *in vivo*. Furthermore, hyporeactivity on *ex vivo* stimulation may indicate that in MS, the CTLA-4-mediated down-regulatory pathway is severely impaired, which may contribute to a failure to terminate ongoing autoimmune response.

DEPARTMENT OF MICROBIOLOGY
Head: Professor Jolanta Zakrzewska-Czerwińska, Ph.D.

Laboratory of the Molecular Biology of Microorganisms
Head: Professor Jolanta Zakrzewska-Czerwińska, Ph.D.
The molecular basis of replication and gene expression and the design of compounds inhibiting these processes

Initiation of bacterial chromosome replication

Bacterial chromosome replication is mediated by a single initiator protein, DnaA, that interacts specifically with multiple DnaA boxes located within the origin (*oriC*). Using *in silico* methods (DNA asymmetry, DnaA box distribution, *dnaA* gene location), the putative *oriC* regions have been identified in 120 chromosomes.

The *Mycobacterium tuberculosis oriC* region contains 13 non-perfect DnaA boxes. The *M. tuberculosis* initiator protein (DnaA) was overexpressed in *E. coli* as a soluble His-tagged fusion protein. The purified protein 6HisMtDnaA was investigated for its binding properties to DnaA boxes from the *oriC* region. Gel retardation demonstrated that the DnaA from *M. tuberculosis* requires two DnaA boxes for efficient binding. Electron microscopy as well as DNase I footprinting showed that the 6HisMtDnaA protein binds to four specific regions, which correspond to the locations of 11 of the 13 previously identified DnaA boxes within the *M. tuberculosis oriC*. The DnaA molecules in *M. tuberculosis* probably, by cooperative binding of numerous 'non-perfect' DnaA boxes, assemble along the *oriC* region and subsequently form a massive nucleoprotein complex.

We also compared the architectures of the DnaA-origin complexes of evolutionarily distantly related eubacteria: two Gram-negative organisms, *Escherichia coli* and *Helicobacter*

pylori, and two Gram-positive organisms, *Mycobacterium tuberculosis* and *Streptomyces coelicolor*. Their origins vary in size (from approx. 200 to 1000 bp) and number of DnaA boxes (from 5 to 19). The presented results indicate that: (i) different DnaA proteins exhibit various affinities toward a single DnaA box; (ii) the spatial arrangement of two DnaA boxes is crucial for the *H. pylori* and *S. coelicolor* DnaA proteins, but not for *E. coli* and *M. tuberculosis* proteins; and (iii) the *oriC* regions are optimally adjusted to their cognate DnaA proteins. The primary functions of multiple DnaA boxes are to determine the positioning and order of assembly of the DnaA molecules. A gradual transition from sequence-specific binding of the DnaA protein to binding through cooperative protein-protein interactions seems to be a common, conserved strategy to generate oligomeric initiator complexes bound to multiple sites.

Laboratory of Signaling Proteins
Head: Associate Professor Wojciech Gorczyca, Ph.D.
Studies on proteins involved in activation of proinflammatory transcription factors in immune cells

We have studied the influence of cGMP-dependent pathways on the activity of NF-κB and AP-1 in human peripheral blood mononuclear cells (PBMCs) and in rat peritoneal macrophages (rPMs). In PBMCs, cGMP was elevated in response to donors of NO, which are known activators of soluble guanylyl cyclases (sGC). On the other hand, rPMs responded with cGMP synthesis only to atrial natriuretic factor (ANF), which is an activator of particulate guanylyl cyclase A (GC-A). We also found that PBMCs expressed cGMP-dependent protein kinase 1 (PKG1), while rat macrophages did not. The activity of NF-κB and AP-1 was differently affected in PBMCs and rPMs by elevation of the intracellular content of cGMP. Therefore, we concluded that cGMP may influence the activity of both proinflammatory factors but the effect depends on the cGMP signaling pathway available in a particular cell type.

DEPARTMENT OF CANCER IMMUNOLOGY
Head: Professor Paweł Kisielow, Ph.D.

Laboratory of Transgenesis and Lymphocyte Biology
Head: Professor Paweł Kisielow, Ph.D.
Development of T cells: identification and characterization of the expression and function of the new genes

We studied NWC, a third transcriptionally active gene within RAG locus, and made the surprising observation that it is ubiquitously expressed, but is differently regulated in lymphocytes than in other cells. In developing T and B lymphocytes it is dependent on the activity of RAG1 promoter and is transcribed together with RAG1. As a result, presumably due to the differential splicing of hybrid RAG1/NWC pre-mRNA, three hybrid RAG1/NWC mRNAs are generated that differ by the number and composition of NWC exons, one of which, discovered in this study, represents antisense sequence for a portion of the RAG2 coding sequence. Mature T and B lymphocytes and their immature RAG-negative developmental stages do not express NWC. This is because in lymphocytes, both expressing and not expressing RAGs, the NWC promoter, located in the second intron of RAG2, is silenced. In other cells the NWC promoter is active and therefore the majority, if not all, nucleated nonlymphoid cells express NWC. Thus there is a clear antinomy between lymphocytes and other cells as to the expression pattern of genes within the RAG locus. In non-lymphocytes, RAGs are silenced but NWC is expressed, while in lymphocytes, either both (i.e. RAGs and NWC) are expressed or both are silenced. This observation suggests that the mechanisms keeping RAGs inactive in lymphocytes and in other cells are different, at least at the chromatin level. In non-T, non-B lineage cells, in which the activity of the NWC promoter is allowed or required, RAGs are selectively repressed, while in RAG-negative lymphocytes, in which the NWC promoter is permanently inactivated, RAGs are repressed together with NWC.

The above findings open new questions with regard to the regulation of expression of RAGs during lymphocyte development and with regard to the mechanisms controlling transcription within the RAG locus in lymphocytes versus non-lymphocytes. Moreover, our results invite speculation on the biological significance of the fact that in lymphocytes the function of the NWC promoter is taken over by the RAG1 promoter.

Laboratory of Cellular and Molecular Immunology
Head: Professor Leon Strządała, Ph.D.
Normal and pathological development and selection of lymphoid and neuronal cells

The orphan nuclear receptors Nur77 (also termed NGFI-B, TR3, NR4A1) and Nurr1 (NR4A2) are the members of the Nur77 family belonging to the nuclear hormone receptor superfamily of transcription factors (Nuclear Receptors Nomenclature Committee, 1999). The Nur77 and Nurr1 proteins are mediators of the cellular response to various external stimuli, leading to proliferation, differentiation, apoptosis, and synthesis of steroid hormones,

depending on the cell type. In particular, Nur77 mediates signals to T cell receptor-induced apoptosis of thymocytes during the stage of T-cell development at which interaction with antigen triggers a negative selection of potentially autoreactive T cells. In contrast, Nurr1 was found to be absolutely required for the development of midbrain dopaminergic neurons.

We demonstrated that the transactivation activity of Nur77 discriminates between Ca^{2+} and cAMP signals. Transcription of the *Nur77* family genes was upregulated in PC12 cells following incubation with Ca^{2+} ionophore as well as cyclic AMP (cAMP) analog. On the other hand, the cAMP analog induced a strong increase, while Ca^{2+} ionophore induced a weak increase, in the transactivation activity of Nur77. We found that Nur77 and Nurr1 proteins were expressed in the nucleus following stimulation with cAMP analog, but not after stimulation with Ca^{2+} ionophore. However, expression of Nur77 protein was increased in the cytoplasm of cells treated with Ca^{2+} ionophore. In conclusion, our results suggest that cAMP-induced and Ca^{2+}-induced processes may differentially regulate the activity of Nur77 at the level of translocation of Nur77 protein from the cytoplasm into the nucleus.

DEPARTMENT OF INFECTIOUS DISEASE MICROBIOLOGY
Head: Professor Andrzej Gamian, Ph.D.

Laboratory of Medical Microbiology
Head: Professor Andrzej Gamian, Ph.D.
Studies on the pathogenesis of some autoimmune diseases of bacterial etiology and the role of sialic acid, glycolipids, endotoxins, and bacterial proteins

Current activity of the laboratory is focused on studies of the pathogenicity mechanisms of diseases of bacterial etiology, the roles of molecular mimicry, bacterial proteins, and glycolipids in pathogenicity, and the structures and functions of bacterial capsular antigens and endotoxins.

The general strategy for elaborating the protective tools against invading bacteria involves the determination of the structures of the molecules involved in infection and immune processes, in probiotic mechanisms, and their chemical and genetic manipulations, as well as understanding their biological activities. Thus the structures of several such antigens have been established. The interference of some bacterial components with functions of tissue structures may contribute to the mechanisms of pathogenicity. Studies on structure of glycolipids from pathogenic actinomycetal microorganisms allow to use these glycolipids for identification and as chemotaxonomic and immunodiagnostic markers useful for the classification and identification of clinical isolates and the recognition of opportunistic

actinomycete as well as for nocardiosis-like infections. In this study the antigenic specificity was established of three similar glycolipids of dimmanosyl diglyceride type differing only in their fatty acid substitutions on the glycerol residue. Glycolipid markers were also useful in the description of a clinical isolate as a new species. The monitoring of specific markers for sepsis and septic shock could significantly facilitate the prognosis of these diseases and their treatment. Consequently, a biosensor for the detection of endotoxins has been prepared. Moreover, studies are continuing on the method of determination of endotoxins as markers for the control of different stages of septic shock and the clinical status during treatment.

DEPARTMENT OF IMMUNOCHEMISTRY
Head: Professor Czesław Ługowski, Ph.D.

Laboratory of Microbial Immunochemistry and Vaccines
Head: Professor Czesław Ługowski, Ph.D.
Biochemical characteristics of macromolecules involved in immunological processes
Immunochemical studies of bacterial endotoxins

Our studies in the year 2004 were the continuation of the immunochemical characterization of endotoxins isolated from prominent opportunistic pathogens, such as *Hafnia alvei* and *Citrobacter*. These bacteria cause typical nosocomial infections, sometimes resulting in severe complications such as bacteremia and endotoxic shock. It has been found that multidrug resistance is widespread among enteric bacteria. This significant finding, in connection with data on the sharing of virulence-associated properties at the phenotypic and genetic levels among enteropathogenic *Escherichia coli, H. alvei*, and *Citrobacter*, led to the conclusion that all of these bacteria should be considered as important diarrhogenic pathogens. Lipopolysaccharide (LPS) is a major virulence factor of these bacteria. It is composed of a lipid A part and a heteropolysaccharide, which generally consists of a core oligosaccharide and O-specific polysaccharide (PS). The O-specific PS defines the serogroup specificity and is important for the biological and physical properties of the overall LPS and plays a significant role in the interaction with the host.

We reported structural and immunochemical studies of the O-specific polysaccharides isolated from the *H. alvei* strain PCM 1189, 1200, 1203, 1529, and *C. youngae* PCM 1538. These structures were investigated by ^{1}H and ^{13}C NMR, MALDI-TOF mass spectrometry, methylation analysis, and immunological methods.

The high molecular mass O-specific PS isolated by mild acid degradation of *C. youngae* LPS of PCM 1538 strain is a homopolysaccharide of 4-acetamido-4,6-dideoxy-D-

mannose (D-perosamine). Two structurally different polysaccharide populations were identified, consisting of sugar units α(1-2) or α(1-3)-β(1-3) linked. *H. alvei* strain PCM 1529 produces branched, acidic O-specific polysaccharide composed of O-acetylated pentasaccharide repeating units. The pentasaccharide consists of three rhamnose residues: terminal, 3-substituted and 2,3-disubstituted, 4-substituted galacturonic acid, and 3-substituted N-acetylglucosamine.

We performed structural and serological studies on O-antigens isolated from two *H. alvei* strains, PCM 1200 and PCM 1203, serologically closely related to PCM 1205. It was demonstrated that both O-deacetylated polysaccharides possessed the same composition and sequence as the O-deacetylated PS of strain PCM1205, that is a branched, glycerol teichoic acid-like polymer. The presence of different epitopes in the O-specific polysaccharides of *H. alvei* strains PCM 1200, 1203, and 1205 could be explained by a variation in the number of O-acetyl groups and their location, established in NMR studies. The structural features of the isolated O-specific PS were also the same as those of the O-specific PS on the bacterial cells directly observed by the HR-MAS NMR technique. We also established the structure of the O-polysaccharide of *H. alvei* strain PCM 1189, which has hexa- to octasaccharide repeating units owing to incomplete glucosylation. The repeating units also include minor O-acetyl groups.

(In collaboration with the Swedish University of Agricultural Sciences, Uppsala, Sweden, and the N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow, Russia).

Laboratory of General Immunochemistry
Head: Associate Professor Maria Janusz, Ph.D.
Studies on the mechanism of action of proline-rich polypeptide (PRP)

Effect on nitric oxide synthase

A proline-rich polypeptide (PRP) complex has not only immunotropic but also procognitive properties. In the form of orally administered tablets (Colostrinin®) it improves the outcome of Alzheimer's disease patients. The positive therapeutic effects of Colostrinin® could be connected with a regulatory effect on the secretion of nitric oxide, reactive oxygen species, and cytokines. We showed that PRP complexes regulate the secretion of an array of cytokines, inhibit production of NO and O_2^- induced by LPS in mice, and inhibit the early steps of both the phenotypic and functional differentiation of cells. In the case of the human monocytic/macrophage THP-1 cell line, widely used as a model of human microglial cells, a

nonapeptide (NP) fragment, but not a whole PRP, complex showed inhibitory activity on the release of NO induced by LPS. Although LPS induced the expression of inducible nitric oxide synthase (iNOS) in THP-1, it was not changed in the presence of PRP and NP. In experiments *in vivo*, mice were intraperitoneally pretreated with LPS in the absence and in the presence of PRP and the induction of iNOS was evaluated in the ascitic fluid cells composed of monocytes/macrophages and lymphocytes. In both cells types LPS, but not PRP, induced iNOS: However, the peptide complex significantly attenuated expression of iNOS induced by LPS.

The results obtained suggest that neither PRP nor NP induces production of NO. However, PRP could regulate both the function and expression of iNOS. This effect, among others, could explain positive therapeutic effect of PRP/Colostrinin® in patients with Alzheimer's disease.

Laboratory of Glycobiology
Head: Professor Maciej Ugorski, Ph.D., D.V.M.
Study on the structure and functions of cell adhesion molecules

α1,3/4-fucosyltransferase (Fut3) is involved in the synthesis of sialyl Le^a tetrasaccharide, a tumor-associated carbohydrate antigen. Fucolsyltransferases are thought to be important regulatory enzymes in the synthesis of fucosylated structures; however, there are conflicting data on the role of Fut3 in the synthesis of this carbohydrate structure. To approach this problem, more studies on the regulation of *FUT III* gene expression are needed. Therefore, as a first step the promoter of *FUT III* was cloned and characterized. Sequencing data showed the absence of canonical TATA, CAAT, and GC boxes, but many binding sites for transcription factors, described in colon cancer cells, were identified. Analysis of enhancer and silencing elements of deletion mutants revealed the presence of basal promoter elements of the *FUT III* gene in the region -854 to -636 bp from the translational initiation site, and a strong negative regulatory element within the -1219 to -1972 bp region. 5'-RACE analysis showed the presence of two transcripts with 5'-ends localized within exon A. The 5'-end of the longer transcript extended -229 nucleotides from the translation start codon and contained a sequence corresponding to an Inr element, localizing the putative transcription initiation site within this sequence. The strong correlation between the promoter activity of *FUT III* and the high expression of sialyl Le^a found in different colon carcinoma cell lines seems to confirm the important regulatory role of Fut3 in the synthesis of sialyl Le^a.

Laboratory of Glycoconjugate Immunochemistry
Head: Associate Professor Hubert Krotkiewski, Ph.D.
Immunochemical and genetic studies on human glycophorin and other proteins active in the immune system

We showed previously that the affinity constant of the recombinant Fab fragment N92, recognizing glycophorin A, is about 75 times lower than that of the monoclonal antibody N92, while the affinity constant of the recombinant Fab fragments, derived from the light chains, is similar to that of N92 antibody. Using point-directed mutagenesis we showed that glycine at position 91 of the light chain plays an important role in antigen recognition. In particular, substitution of Gly-91 by serine decreases the affinity by a factor of 50. Edman sequencing of the N-terminal fragments of the light chains revealed that there is an additional alanine in the N-terminus. This alanine is probably a remnant of the OmpA signal peptide, which was cleaved at a wrong place by *E. coli* signal peptidase. In addition, we found that threonine at position 5 was replaced by glycine. Taken together, all these changes caused a significant decrease in the N92 recombinant Fab fragment. The high-affinity, library-derived NNA7 fragment has been purified and crystalized, and now is being analyzed using X-ray diffraction. The preliminary data suggest a pivotal role of glycine at position 91.

Serum IgG is a characteristic marker in rheumatoid arthritis, i.e. it lacks galactose residues in N-glycans, present in Fc fragment, proportionally to the severity of the disease. We have measured this agalactosylation using three independent methods: 1) total sugar analysis (GLC-MS after sample hydrolysis), 2) ELISA, using two biotinylated lectins: RCA-I and GSL-II, and 3) biosensor BIAcore, using the same two lectins as ligands. Based on ELISA results we have calculated the *agalactosylation factor* (AF), which helps to compare different IgG samples. Using biosensor BIAcore we analyzed the galactosylation of IgG, derived from patients during a combined therapy, employing methotrexate. It was shown that the described treatment of RA patients produces a partial reconstitution of the conservative N-glycans galactosylation, which also correlates with more positive clinical symptoms of the disease.

Myelin basic protein (MBP) and myelin proteolipid protein (PLP) are the main components of myelin, accounting for about 30% and 50% of total myelin proteins, respectively. MBP and PLP are the major candidate autoantigens in multiple sclerosis (MS), a human autoimmune disease, because T cells specific to MBP and PLP were isolated from the cerebrospinal fluid and peripheral blood of MS patients. Therefore we aim at the preparation and immunochemical characterization of the recombinant forms of both myelin proteins. Plasmids containing cDNA coding for MBP (21.5-kDa isoform) and PLP (26-kDa classic

form) were obtained from Dr. A. Campagnoni (UCLA, CA, USA). Two eukaryotic expression pCDNA3 vectors containing cDNA for MBP and PLP, respectively, were prepared and used for transient transfection (by electroporation) of COS7 cells. The expression and cytoplasmic localization of MBP and the surface expression of PLP were shown in confocal scanning microscope. Because of the low-level expression of MBP and PLP (15-20%) in COS7 cells, we intend to obtain stable clones of CHO cells expressing both proteins.

DEPARTMENT OF MEDICAL IMMUNOLOGY
Head: Professor Andrzej Górski, M.D.

Laboratory of Bacteriophages
Head: Professor Andrzej Górski, M.D.
Application of specific bacteriophages in the treatment of bacterial infections and their possible role in host defense and disease

Bacteriophages (phages) as bacterial viruses are generally believed to have no intrinsic tropism for mammalian cells. In the Bacteriophage Laboratory the interactions between phages and various eukaryotic cells were investigated. Previously we observed binding of T4 phage to the membranes of cancer and normal blood cells. We selected a mutant: HAP1 with enhanced affinity for melanoma cells. Both T4 and HAP1 markedly and significantly inhibited experimental lung metastasis of murine B16 melanoma, and HAP1 was more effective than T4 in this.

The potential phage anticancer activity was then investigated in primary tumor models (B16 melanoma, s.c.). Treatment with purified preparations of bacteriophage T4 resulted in significant reduction in tumor size; HAP1 was more effective than T4. The effect was dose-dependent. Parallel experiments with unpurified bacteriophage lysates resulted in significant stimulation of tumor growth. These data suggest that purified bacteriophages may inhibit tumor growth and highlight the importance of efforts on the improvement of bacteriophage purification procedures. Endotoxins possess a high degree of toxicity *in vitro* and *in vivo*, and their removal is essential for safety in antibacterial bacteriophage therapy. An effective, scaleable purification of bacteriophages from endotoxins was accomplished by sequential ultrafiltration through polysulfone membrane followed by chromatography on sepharose 4B and Matrex Cellulofine Sulfate. The phage fraction after gel filtration chromatography routinely contained endotoxins in the 150-2500 EU/ml range. The procedure yielded bacteriophages contaminated with as little as 0.4-7 EU/ml (Limulus assay). This value lies

within the permitted level for intravenous applications (5 EU/kg/h by European Pharmacopoeia, 1997).

Investigating the molecular mechanisms of phage-eukaryota interactions, we found a mutation in the *hoc* gene that differentiates bacteriophage HAP1 and its parental strain T4. The detected mutation is a non-sense type and occurs at 44% of *hoc*'s length. We found that the head of HAP1 is smaller than that of T4 (by the electron micrographs and by dynamic light scattering). This is in line with the well-described morphogenesis of the T4 capsid: after incorporation of Hoc protein, the T4 phage head becomes visibly larger. These results indicate that HAP1 lacks gp Hoc. The normal Hoc protein is balloon-shaped and it extends to about 5 nm away from the capsid surface, with 160 regularly arranged units per capsid. Because of its special localization, gp Hoc impedes access of external factors to the head surface. Without Hoc there are no important spatial disturbers that can diminish the interactions of other head components with any external targets. This also applies to gp 24, which was proposed as the active protein.

Hoc protein is necessary neither for T4 viability nor for its structure, and its exact function is unknown. Our results suggest that some bacteriophage molecules are predicted to interact with eukaryotic organisms and/or to modulate these interactions. Hoc protein seems to be one of these molecules.

Laboratory of Cellular Interactions
Head: Associate Professor Danuta Duś, Ph.D.
New markers of tumor progression. Cancer cell-endothelial cell interactions during metastatic spread of cancer cells. Organ specificity of metastasis.

The variable outcome of cancer patients with similar clinical status creates a need of searching for new, reliable prognostic indicators of tumor progression, recurrence, and survival. The aim of the study was to determine the prognostic value of several new molecular traits of tumor cells in laryngeal, urinary bladder, kidney, and breast cancers which determine tumor progression and metastatic growth. In squamous cell carcinoma of the larynx, the expressions of c-myc oncoprotein and Bcl-Xl protein were evaluated. Tumor specimens from 50 patients with carcinoma of the larynx were evaluated immunohistochemically. The results suggest that Bcl-Xl protein, but not c-myc oncoprotein, expression may be of some value in predicting the clinical course of patients with laryngeal cancer. The prognostic value of flow cytometric evaluation of DNA ploidy and tumor cell proliferative activity in surgical biopsies of laryngeal and kidney tumors were examined. In

laryngeal cancer, positive correlation between DNA aneuploidy, presence of nodal metastases, and S+G2M phase fraction was documented. In renal cancer, aneuploidy in combination with clinical stage T>2 and the presence of a high fraction of proliferating tumor cells indicated increased probability of tumor progression. Soluble intercellular adhesion molecule-1 (sICAM-1) level was examined in renal cancer patients. It has been observed that preoperative high sICAM-1 level (<221 ng/ml) was connected with a high probability of post-operative disease progression and metastatic tumor growth. It has been hypothesized that tumor cells expressing Fas (death receptor; CD95) and its ligand (FasL) are able to avoid immune surveillance. An examination of the level of Fas/FasL expression in tumor tissue of 130 patients of the Lower Silesian Oncology Center with stage II breast carcinoma was performed (in the collaboration with Marek Bębenek, M.D.) The results, analyzed after three years of follow-up, indicated that Fas presence on human breast cancer cells significantly correlated with a lower rate of lymph node involvement, longer disease-free time, and survival.

During blood-borne metastatic spread, extravasation of tumor cells is a prerequisite for distant tissue colonization. Necessary for this extravasation are close adhesive interactions of the cancer cell surface molecules with endothelial cells at the site of cancer cell extravasation. Our studies on endothelial cells are aimed at their phenotypic characteristics, particularly their activation mechanisms and organ-specific adhesive interactions under normal and pathological conditions. Among others, we reported, for the first time, the presence of an IL-7 functional receptor on human microvascular endothelial cells. A study on the adhesion of lymphocytes from children with asthma to endothelial cells of different tissue origin revealed that B lymphocytes have significantly greater adhesive potential towards endothelial cells from lungs and skin than those of other origin. We also evaluated the presence of trophoblasts and soluble adhesion molecules: VCAM-1 and ICAM-1 in the peripheral blood of women with pregnancy-induced hypertension (PIH). The results indicate a correlation between increased trophoblast number and higher levels of VCAM-1, an endothelium activation marker, and the occurrence of PIH. Studies on endothelial cells were performed in collaboration with C. Kieda, Ph.D., from CBM CNRS, Orleans, France.

Laboratory of Virology
Head: Professor Zofia Błach-Olszewska, Ph.D.
Study on nonspecific immunity in viral infection

Innate immunity of peripheral blood leukocytes isolated from patients with acute leukemias was compared with the immunity leukocytes of healthy blood donors. An individually differentiated degree of innate immunity in both groups was found. Statistically significant lower innate immunity in leukemic patients was shown. A dependency of remission (after chemotherapy) and survival time on the degree of innate immunity was noticed. In contrast to patients with a deficiency in innate immunity, who all died, all patients with good immunity had long-lasting remission, all are still alive, and improvement of clinical state was achieved. The level of innate immunity at diagnosis could be considered as a prognostic factor in respect to the induction of remission and survival of patients with acute leukemias.

Despite being exposed to HIV, some persons remain uninfected. The CCR5 genotype effect on progression of HIV-1 infection was studied. In a control group consisting of healthy, uninfected, unexposed individuals, the frequency of the *CCR5-Δ32* allele was found to be 13.4%. In HIV-1-infected patients a significantly lower frequency was observed (7.6%). In contrast, among exposed uninfected persons a 20.6% frequency of the gene was found. The observed differences were significant. This strongly suggested that the actual role of the *CCR5-Δ32* mutation in sensitivity to HIV-1 infection was much more meaningful than that described on the basis of American and Asian population studies.

Additionally, the antiviral activity of thirty new analogues of ebselen were tested. Some of them were found in the antiviral assay *in vitro* to be strong inhibitors of the cytopathic activity of herpes simplex virus type 1 (HSV-1) and encephalomyocarditis virus (EMCV).

Nitric oxide in viral infections coexisting with bronchial asthma was also studied. Airway inflammation in asthmatics during exacerbations with coexisting respiratory viral or mycotic infections was found to be associated with an increased production of nitric oxide (NO) despite chronic treatment with inhaled corticosteroids. Airway neutrophils, in response to rhinovirus or fungal cell-wall constituent, respectively, are supposed to be involved in the process.

Laboratory of Reproductive Immunology
Head: Associate Professor Anna Chełmońska-Soyta, V.D.
Immunological mechanisms associated with reproductive processes in health and disease

Cytokines in endometriosis

Endometriosis, a common disease in women of reproductive age, is characterized by the presence of endometrial tissues outside the uterus. Dysmenorrhea, pelvic pain, and infertility

are the main clinical manifestations of the disease. An immunological basis has been considered to be important in the pathogenesis of endometriosis. We aimed to study T-helper (Th1 and Th2) cytokine levels by Becton Dickinson Cytometric Bead Array Assay by flow cytometry in the peritoneal fluid (PF) of women with endometriosis in four stages of disease according to AFS classification. The results were compared with the cytokine levels in PF of women with idiopathic infertility and pelvic pain syndrome. We observed that the levels of the examined cytokines did not correlate with the progression of the endometriosis. The increasing level of IFN-γ with concomitant decreasing levels of IL-10 and IL-6 in the PF of women with IV stage and the increased IFN-γ/IL-10 and IFN-γ/IL-4 ratios indicate a Th1 response predominance in IV stage of endometriosis, while stages I, II, and III are under the control of Th2 lymphocytes. The levels of all the examined cytokines except IL-6 were significantly higher in the infertility group then in women with stage I endometriosis. The levels of g-IFN and IL-4 were significantly higher in patients with PPS in comparison with stages I and II; however, there were no differences in cytokine levels between PPS women with stage III and stage IV of the disease. We concluded that the determination of pro-and anti-inflammatory cytokine levels in PF may be a valuable diagnostic tool in the diagnosis of infertility.

Tubal infertility

There are numerous sperm receptors involved in the process of gamete recognition. One is the mannose ligand receptor displayed after the capacitation process. It predominantly occurs in the Fallopian tubes and this phenomenon is prerequisite for the process of egg fertilization. We examined the changes in the expression of sperm mannose-ligand receptor after capacitation in the supernatants of tubal mucosa cultures stimulated by LPS. The results showed that LPS stimulation of tubal mucosa decreased the expression of the mannose-ligand receptor. This indicates an additional mechanism of impairment of the fertilization process during tubal infection in women.

Laboratory of Tissue Immunology
Acting head: Assistant Professor Beata Nowakowska, Ph.D.
The genetic diversity of the HLA A19 allelic family and rare locus A alleles in the south-western Polish population

We continued our investigation on the population genetics of the HLA system. The goal of our study was to verify, by typing at the DNA level, the allele frequencies of the HLA A19 family. The frequencies of HLA A29, A30, A31, A32, and A33 were first defined in our

population by serological typing in 1992. The second goal was to determine the frequencies of a rare allele from locus A.

A total of 712 unrelated healthy individuals of both sexes living in and around the Wrocław area were included in the study. The frequency of HLA-A* 29 was 0.0225, A* 30 was 0.0337, A* 31 was 0.0407, A* 32 was 0.0630, and A* 33 was 0.0182. Discrepancies were identified between the serologic and DNA assignments for HLA A32 and A33.

>From the rare alleles we typed antigen HLA A* 34 with a frequency of 0.0014, A* 43 (an allele characteristic of the South African Bushman) and A* 74 (thought to be a characteristic allele of the black population) both had the same frequency of 0.0014, and A* 66 (which is structurally related to A34) had 0.0098. We did find the HLA A* 36 allele in our population, which is present in Caucasoid populations with an average frequency of 0.0018.

Genetic predisposition to cancer development

A polymorphism of p53 at codon 72, encoding arginine (arg) or proline (pro) residue, is located in the proline-rich region of p53 protein, a domain important for growth suppression and apoptosis. The two p53 variants have been reported to have different biochemical and functional properties. The significance of this polymorphism with respect to a risk for cancer have been studied for quite long time, but the results remain inconclusive.

A strong correlation is apparent between the p53 codon 72 polymorphism and ethnicity, with the arg allele being prevalent in populations living further away from the equator. It is possible that the phenomenon of uneven allele distribution is further complicated by a differential allele expression in heterozygotes. We evaluated the expression status of codon 72 polymorphic alleles in healthy Polish (Caucasian) and Chinese (Asian) populations (in collaboration with Dr. K. Sabapathy, Laboratory of Molecular Carcinogenesis, National Cancer Center, Singapore). A preferential expression of the arg allele in Polish heterozygote (arg/pro) subjects and the pro allele in Chinese heterozygotes (arg/pro) was observed. The data suggest that codon 72 expression status rather than genotype might be associated with an increased risk for cancer disease.

DEPARTMENT OF CLINICAL IMMUNOLOGY
Head: Professor Andrzej Lange, M.D.

Laboratory of Clinical Immunology
Head: Professor Andrzej Lange, M.D.
Genetic background and pathomorphologic evaluation of the alloreactive reaction following hematopoietic stem cell transplantation (HSCT)

As a continuation of our research on genetic factors affecting the susceptibility to disease we focused on polymorphisms located within the IFN-gamma, HSP70-hom and CCR5 genes and their relationships with the outcome of allogeneic HSCT. The following associations were found:

(i) IFN-gamma 2/2 genotype constituted an independent and protective factor associated with a decreased risk of grade II – IV aGvHD (Mlynarczewska et al. *Bone Marrow Transplant, 2004, 34, 339-344*); (ii) Recipient HSP-AA homozygous genotype was a risk factor for aGvHD. None of donor HSP genotypes or patient-donor incompatibility within HSP alleles was associated with susceptibility to toxic complications or aGvHD (Bogunia-Kubik and Lange, *Transplantation, in press*); (iii) Patients undergoing allogeneic hematopoietic stem cell transplantation having the CCR5delta32 deletion mutation (associated with defective CCR5 expression) less frequently developed aGvHD.

The further activity of our laboratory in the field of HSCT included:

(i) Implementation of autologous bone marrow transplantation procedure for angiogenesis regeneration in patients with ischemic legs. Ten male patients with atherosclerotic critical leg ischemia suffering from pain at rest and/or foot ischemic ulceration, in whom surgical treatments were exhausted were enrolled in this study (Lange et al. *Blood 2004, 104 (11), 131b)*.

(ii) Description of an autologous origin of stromal cells in patients with chronic myeloid leukemia, recipients of allogeneic transplant from PBPC. In this study 4 CML patients were investigated for post transplant chimerism for total marrow cells and mesenchymal stem cell populations purified and propagated from marrow cells. Patients were conditioned with a nonmyeloablative regimen and at the time of investigation for allogeneic HSCT were disease free with proven hematological, cytogenetical and genetical remission.

We also studied malignant ascites and tumors of patients with untreated ovarian carcinoma to examine tumor angiogenesis in the tumor microenvironment. CXCL12 and vascular endothelial growth factor were found to synergistically induce neoangiogenesis (Kryczek et al. *Cancer Res., in press*).

In a separate analysis, IFN-alpha treatment together with anti-cancer therapy was found to be associated with an increased fraction of effector CD4+ memory lymphocytes affecting the tumor cells.

Moreover, in November 2004 the 9th round of the Proficiency Testing of HLA class I Typing for Central-East Europe took place. Our activity in the standardization area was awarded by the European Federation for Immunogenetics during the 18th European Immunogenetics and

Histocompatibility Conference, Sofia, Bulgaria (Bogunia-Kubik and Lange. *Genes Immunol., 2004, 5, suppl. 1, S44*).

Laboratory of Immunogenetics
Head: Associate Professor Piotr Kuśnierczyk, Ph.D.
Association of psoriasis vulgaris with KIR2DS1 gene

Psoriasis vulgaris, particularly its juvenile form, is strongly associated with the *HLA-Cw*06* allele encoding HLA-Cw6 molecule. This molecule is recognized by the inhibitory receptor KIR2DL1 and the activatory receptor KIR2DS1, which are expressed on natural killer cells and subpopulations of T lymphocytes. Humans differ by the presence or absence of particular *KIR* genes. We hypothesized that either the activatory *KIR2DS1* or the inhibitory *KIR2DL1* gene frequencies might be different in psoriatic patients from those of a control population. Therefore we compared the frequencies of *KIR2D* inhibitory (*L*) and activatory (*S*) genes in 116 psoriasis vulgaris patients and in 123 healthy controls. 14 novel gene combinations were found (6 in patients, 7 in controls, and 1 in both). *KIR2DS1* was present in 85% of the patients, but only in 51% of the controls ($pc < 0.0009$). Similarly, *HLA-Cw*06* was much more frequent in patients (77%) than in controls (17%; $pc < 0.00002$). Statistical analysis suggests that although the contribution of these two factors to psoriasis is partially independent, they nevertheless interact. This result strongly speaks for a role of KIR2DS1 upon recognition of HLA-Cw6 in susceptibility to psoriasis.

Description of two new polymorphisms in the FCAR gene and study on their distribution in allergic asthma patients and healthy controls

Eosinophils are important components of allergic inflammation. The IgA Fc receptor (FcαRI), encoded by the *FCAR* gene, is a possible candidate for eosinophil activation on mucosal surfaces, where IgA is abundant. Both elevated cell surface expression of FcαRI and increased avidity for IgA were described on eosinophils from allergic subjects. The aim of our study was to examine the possible association of *FCAR* gene polymorphisms with allergic asthma. We screened three regions of the *FCAR* gene: (i) the promoter region, (ii) exon 3, encoding the first extracellular domain (EC1), and (iii) exon 5, coding for the transmembrane and cytoplasmic domain, for new and published polymorphisms using a sensitive temperature gradient gel electrophoresis technique (TGGE) and compared the frequencies of these polymorphisms in 112 patients diagnosed with allergic asthma and 100 healthy controls. Six polymorphisms, including two novel ones (-340G/A in the promoter region, and 844A/G in exon 5 resulting in a 248Ser/Gly substitution in cytoplasmic region), were detected. No

differences between patients and controls were found in the distribution of any of these polymorphisms. FcαRI polymorphism does not seem to be a risk factor in allergic asthma. Nevertheless, this is a first report on the distribution of 6 single-nucleotide polymorphisms of the *FCAR* gene in a human population and the first study on *FCAR* polymorphism in allergic asthma published so far.

P u b l i k a c j e - 2004

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***Prace przeglądowe opublikowane*:**

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