Phage neutralization by sera of patients receiving phage therapy

The aim of our investigation was to verify whether phage therapy (PT) can induce antiphage antibodies. The antiphage activity was determined in sera from 122 patients from the Phage Therapy Unit (PTU) in Wrocław with bacterial infections before and during PT and in sera from 30 healthy volunteers using a neutralization test. Furthermore, levels of antiphage antibodies were investigated in sera of 19 patients receiving staphylococcal phages and sera of 20 healthy volunteers using the enzyme-linked immunosorbent assay (ELISA). The phages were administered orally, locally, orally/locally, intrarectally or orally/intrarectally. The rate of phage inactivation (K) estimated the level of phages’ neutralization by human sera. Low K rates were found in sera of healthy volunteers (K up to 1.73). Low K rates were detected before PT (K up to 1.64). High antiphage activity of sera K>18 was observed in 12.3% of examined patients (n=15) treated with phages locally (n=13) or locally/orally (n=2) from 15 to 60 days of PT. High K rates were found in patients treated with some S. aureus, P. aeruginosa, and E. faecalis phages. Low K rates were observed during PT in sera of patients using phages orally (K up to 1.04). Increased inactivation of phages by sera of patients receiving PT decreased after therapy. These results suggest that the antiphage activity in patients’ sera depends on the route of phage administration and phage type. The induction of antiphage activity of sera during or after PT does not exclude a good result of PT.

Bacteriophage interactions with phagocytes and their potential significance in experimental therapy

We evaluated whether application of phage preparations impairs bactericidal activities of human phagocytes (granulocytes and monocytes). In our study, we used preparations of phages T2 and T4 specific to Escherichia coli and A3 phage specific to Staphylococcus aureus. We found that bacteriophage preparations do not influence intracellular killing of bacteria by human phagocytes. The effect is irrespective of phage preparation type (lysate,
purified phage preparation), phage titer of the preparation, and whether bacteria phagocytosed by phagocyte cells are sensitive or insensitive to phage (bacteriophages homologous and heterologous to bacteria). Although the results of our study are preliminary, they support previous data indicating safety of therapeutic application of phages.

**Influence of bacteriophage preparations on migration of HI-60 leukemia cells in vitro**

The results we presented also concern the influence of bacteriophages on migration of human leukemia cells (HI-60). Most phage preparations used in our study did not influence migration of HI-60 cells. The only phage preparation which stimulated migration of leukemia cells was Staph.1N/80, specific to *Staphylococcus aureus*. The molecular basis of interactions between bacteriophages and HI-60 cells currently cannot be explained due to extremely scant and contradictory data regarding molecular structure and biology of these viruses. We may assume that the role of phage 12 proteins connected with phage specificity is limited in described interactions as A5 bacteriophage, which has the same host bacteria as Staph.1N, does not influence migration of HI-60 cells. The results of our study indicate that phages do not influence migration of HI-60 cells (the effect is similar to that for phagocytes). There are no data indicating any side effects of phage therapy. The results of our study support previous data indicating that phage therapy may be safe for cancer patients. However, further studies are certainly required.

**Microbiological quantitative bacterial cultures in patients subjected to phage therapy**

The aim of the study was to determine the usefulness microbiological quantitative bacterial cultures for monitoring experimental phage treatment (PT). In the year 2013, a systematic evaluation of the bacterial number in wounds was done in 13 patients with chronic purulent fistulas (8 patients) and infected ulcerations (4 patients) treated at the Phage Therapy Unit in Wroclaw. One of them was infected with both *S. aureus* and *P. aeruginosa*, in 10 patients *S. aureus* was isolated, in two others *P. aeruginosa*, and in another one *E. cloacae*. One patient with chronic *S. aureus* ear was also included in the analysis. Quantitative bacterial cultures were done according to the standard procedures previously established in the Bacteriophage Laboratory (LAP 07, SOP 071, SOP 072, SOP 073). Samples were collected using wet sterile compresses, or swabs, which were immediately immersed into 2 ml of sterile saline. Then they were shaken, and the amount of bacteria in suspension was determined after 48 h culture on blood agar/MacConkey agar plates at 37°C.

At least one log drop of the bacterial titer in the sample taken during or after PT in comparison to the sample taken before PT was considered significant. It was observed in 4 of
6 patients with a good response to PT and only 2 of 7 patients with an inadequate response to PT. However, the differences in these frequencies between the two groups of patients (66% vs. 29% respectively) were not statistically significant (p = 0.29 in Fisher’s exact test). Interestingly, we observed in individual cases that bacterial eradication was not required for wound healing observed during PT. Therefore we plan to continue these studies in a larger group of patients.

**Bacteriophage purification by affinity chromatography**

A novel approach for separating bacteriophages from contaminating bacteriophages of other types using affinity chromatography combined with competitive phage display has been developed. Practical applications of bacteriophages in medicine and biotechnology induce a great need for technologies of phage purification. None of the popular methods offer solutions for separation of a phage from another similar phage. We used affinity chromatography combined with competitive phage display (i) to purify T4 bacteriophage from bacterial debris and (ii) to separate T4 from other contaminating bacteriophages. In ‘competitive phage display’ bacterial cells produced both wild types of the proteins (expression from the phage genome) and the protein fusions with affinity tags (expression from the expression vectors). Fusion proteins were competitively incorporated into the phage capsid. It allowed effective separation of T4 from a contaminating phage on standard affinity resins.

**Studies on induction of inflammatory mediators by bacteriophages and phage proteins**

T4 phage and its head surface proteins were studied as regards stimulatory effects on inflammatory mediator production. Justification for these studies was the fact that viruses are potent activators of the signal pathways leading to increased cytokine or ROS production. The effects exerted on the immune system are usually mediated by viral proteins. Complementary to the progress in phage therapy practice, advancement of knowledge about the influence of bacteriophages on mammalian immunity is necessary. Particularly, the potential ability of phage proteins to act like other viral stimulators of the immune system may have strong practical implications for the safety and efficacy of bacteriophage therapy. Here we present studies on the effect of T4 phage and its head proteins on production of inflammatory mediators and inflammation-related factors: IL-1α, IL-1β, IL-2, IL-6, IL-10, IL-12 p40/p70, IFN-γ, TNF-α, MCP-1, MIG, RANTES, GCSF, GM-CSF and reactive oxygen species (ROS). Plasma cytokine profiles in an *in vivo* mouse model and in human blood cells treated with gp23*, gp24*, Hoc and Soc were evaluated by cytokine antibody arrays. Cytokine production
and expression of CD40, CD80, CD86 and MHC class II molecules were also investigated in mouse bone marrow-derived dendritic cells treated with whole T4 phage particle or the same capsid proteins. The influence of T4 and gp23*, gp24*, Hoc and Soc on reactive oxygen species generation was examined in blood cells using luminol-dependent chemiluminescence assay. In all performed assays, the T4 bacteriophage and its capsid proteins gp23*, gp24*, Hoc and Soc did not affect production of inflammatory-related cytokines or ROS. These observations are of importance for any medical or veterinary application of bacteriophages.

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

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

The purpose of the study was to produce, in insect cells, a recombinant binding region of EBA-140 ligand of *Plasmodium falciparum*. Baculovirus-mediated expression of the recombinant proteins is a valuable method to produce soluble and active proteins, especially with a high content of disulfide bridges. We performed large-scale expression of the recombinant myc/His-tagged EBA-140 region II, cloned from genomic DNA of *P. falciparum* Dd2 strain, using a baculovirus expression system (BD BaculoGold). The SF9 cell culture was harvested 50 h post infection and supernatant fluid was collected. The presence of His-tag at the C-terminus of region II allowed its one-step purification by affinity chromatography on Ni-NTA agarose. We obtained about 4 mg of the recombinant protein from one liter of cell culture. The correct molecular mass, purity and secondary structure of the recombinant EBA-140 region II was analyzed using analytical size exclusion chromatography on a Superdex 200 column and circular dichroism (CD).

VHs, also known as Nanobodies™, are recombinant fragments of heavy-chain variable domain of camel antibodies. The aim of this study was to perform characterization of VH antibody, named IH4, that recognizes human glycophorin A (GPA) from the membranes of human erythrocytes. IH4 recognizes GPA on red blood cells independently of MN blood group status of the molecule. Analysis of IH4 antibody binding to the peptides synthesized on the polypropylene pins showed that it recognized a linear epitope peptide corresponding to amino acid sequence Y52PPE55 of the polypeptide chain of glycophorin A. It was also shown that the protein construct, obtained by fusion of HIV p24 to monovalent VH-IH4, added to the blood of HIV-positive patients, resulted in agglutination of erythrocytes. Since the IH4
antibody recognized the red blood cells of all humans except very rare En(a-) erythrocytes, it may be a useful autologous agglutination reagent.

Laboratory of General Immunochemistry
Head: Professor Maria Janusz, Ph.D.

Studies on the mechanism of action of a proline-rich polypeptide complex (PRP)

Effect on IL-6 release and nerve growth factor (NGF) production in human U373 astrocytoma cell line

Proline-rich polypeptide complex (PRP) shows multidirectional activity, affecting the immune and nervous system. Its positive clinical effect was shown in the case of Alzheimer’s disease (AD) when applied to the patients in the form of Colostrinin® tablets. In AD pathogenesis the main role is played by astrocytes and microglial cells. In astrocytes in the presence of amyloid β (Aβ) deposits the increased secretion of IL-6 was observed. So, the modulatory effect of PRP on IL-6 secretion in human astrocyte cell line U373 induced with Aβ42 peptides was investigated. In preliminary experiments no satisfactory level of IL-6 was observed in the presence of Aβ used as a cytokine inducer. For this reason an astrocyte line with CD14 overexpression (U373+R) was used. It was found that PRP is a weak IL-6 inducer. It was also observed that the level of IL-6 secreted after LPS induction was lowered when cells were preincubated with PRP.

Nerve growth factor (NGF) synthesized by astrocytes is important for nerve cell differentiation. Its level is lowered during senescence and in the case of AD. In astrocyte line U87 and U373+R it was found that in the presence of TNFα (reference inducer), PRP and one of its constituent peptides, VESYVPLFP (NP), NGF release is induced. For example, in U87 cells with use of TNFα, PRP and NP 11.7 pg/ml, 6.26 pg/ml and 14.51 pg/ml of NGF was released, respectively.

The results obtained indicate that both regulation and NGF secretion can be additional neuroprotective effects of PRP/NP in the case of AD.

Results of grant activities

Proline-rich polypeptide complex (PRP) and its nonapeptide fragment (NP) influence neuritogenesis and protect against toxic effect of amyloid β 1-42

The protective effect of proline-rich polypeptide (PRP) and its nonapeptide constituent (NP) on neuronal cells in the presence of Aβ peptides 1-42 was studied. It was found that PC12 cells treated with toxic doses of Aβ42 survive much better when cultured in the presence of PRP or NP. It was also found that the intracellular level of NO induced by Aβ42
was lowered when PC12 cells were treated with PRP/NP. The lack of relationship between NO secreted and the level of NOS suggested that peptides influence enzyme activity rather than its level. The effect of PRP/NP on intracellular NO induced with Aβ42 did not influence cGC or cGMP level.

Studies on the transcriptional regulation of the gene encoding the human neonatal Fcγ receptor (hFcRn)

In 2013, the fifth step of the grant project was performed. The aim of this study was to evaluate the contribution of the identified binding sites for transcription factors in the transcriptional regulation of the $hF\text{c}Rn$ gene. Site-directed mutagenesis and transient transfection assays showed that: Ap1, Sp1, CF1/YY1 binding sequences within the hFcRn promoter are involved in regulation of the $hF\text{c}Rn$ transcription in the human THP1 (differentiated to macrophages), Caco-2, Lu106, HUVEC cells. The binding sites Ap1 at position -276, Sp1 at -313, and CF1/YY1 at -584 have a strong positive effect on hFcRn promoter function. The Sp1 regulatory sequence at positions -643 and -635 (the binding site for transcription factor Sp1 family: Sp1, Sp2, Sp3) acts as an activator and repressor of the $hF\text{c}Rn$ transcription in all the studied cells. The CF1/YY1 motif at position -357 activates the hFcRn promoter in the human Caco-2, Lu106, HUVEC cells whereas in THP1 it functions as a negative regulatory element. Treatment of the Caco-2, Lu106, HUVEC cells with LPS or PMA caused a 2-fold increase of the hFcRn promoter activity compared with that in unstimulated cells. When these cells were transiently transfected with the mutant promoter construct containing the mutation at position -497 or -233, and then were stimulated with LPS or PMA, the hFcRn promoter activity was reduced almost to the same level as that of the wild type promoter construct in untreated cells. These data taken together suggest that the binding sites for the C/EBP family of transcription factors (C/EBPβ, C/EBPδ) located within the hFcRn promoter at positions -497 and -233 function as positive regulators of $hF\text{c}Rn$ gene transcription under pathophysiological conditions.

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

Enterobacterial common antigen (ECA) is a surface antigen present in Gram-negative bacteria belonging to the Enterobacteriaceae family, including Escherichia coli, Salmonella spp., Shigella spp., Klebsiella spp., Enterobacter spp., Citrobacter spp., Serratia spp., Proteus
spp., *Yersinia* spp., and *Plesiomonas shigelloides* (1). These bacteria are responsible for healthcare-associated infections, such as intestinal infections and nosocomial infections (e.g., sepsis). Drug resistance in a few members of this family, notably *Escherichia coli*, *Klebsiella* spp. and *Proteus* spp., is an increasing global problem. This stresses the need for new cross-protective vaccines or therapeutic strategies against Gram-negative bacteria. Some of these could be based on ECA.

ECA is heteropolysaccharide built from the trisaccharide repeating unit $\rightarrow 3)\alpha-D\text{-}Fucp4\text{NAc}-(1\rightarrow4)\beta-D\text{-}\text{ManpNAcA}-(1\rightarrow4)\alpha-D\text{-}\text{GlcNAc}-(1\rightarrow$, modified with O-acetyl groups. The biological significance of ECA is not fully understood and requires further study. We have presented the first structural evidence for the existence of ECA$_{\text{LPS}}$ through a structural analysis of *S. sonnei* phase II LOS preparations. More precisely, we have identified the covalent linkage between ECA and LPS. *S. sonnei* phase II expresses the rough form of endotoxin – LOS with the core OS of *E. coli* R1-type and devoid of O-specific PS. We have found that this ECA$_{\text{LPS}}$ is a molecule consisting of the LOS substituted with one to four repeating units of ECA in the outer core region. It coexists as $\sim 3\%$ of the total amount of poly- and oligosaccharides released after mild acid hydrolysis of *S. sonnei* phase II LOS, indicating that unsubstituted core oligosaccharides prevail on the bacterial surface. In identifying the covalent linkage between $\rightarrow 4)\alpha-D\text{-}\text{GlcNAc}-(1\rightarrow$ of ECA and $\rightarrow 3)\beta-D\text{-}\text{Glc}p$ of the outer core OS, we have also revealed a biological repeating unit of ECA$_{\text{LPS}}$ identical to that present in ECA$_{\text{PG}}$, an expected outcome given the biosynthesis of ECA. The number of repeating units was detected using ESI-IT-MS analysis. Because this MS technique has a limited $m/z$ detection range, we attempted to search for longer polymers of ECA$_{\text{LPS}}$ using MALDI-TOF MS. Unfortunately, this technique did not produce good quality spectra, even for the shortest glycoform, [ECA]-dLOS. However, the possibility that longer polymers of ECA$_{\text{LPS}}$ are present cannot be excluded. Among the modifications reported previously for ECA$_{\text{CYC}}$, including O-acetylation and a nonstoichiometric lack of N-acetyl groups in the case of $\rightarrow 4)\alpha-D\text{-}\text{Glc}p\text{NAc}-(1\rightarrow$ residue, only O-acetylation was observed for *S. sonnei* ECA$_{\text{LPS}}$. Moreover, one O-acetyl group was identified only in fraction III, consisting of the core OS substituted with four repeating units of ECA.

Notably, in the structure presented, ECA occupies the position that used to be substituted with the O-specific PS, $\rightarrow 4)\alpha-L\text{-}\text{Alt}p\text{NAcA}-(1\rightarrow3)\beta-D\text{-}\text{FucpNAc4N}-(1\rightarrow$ in the case of smooth *S. sonnei* phase I. This finding may exclude the simultaneous presence of the O-antigen and ECA within the same glycoform of the core OS. However, the coexistence of core OS glycoforms with ECA or O-specific polysaccharides on the surface of smooth
*Enterobacteriaceae* is possible since most bacteria synthesize significant amounts of unsubstituted core OS.

It should be emphasized that there are obvious inherent difficulties in the structural analysis of ECA\textsubscript{LPS}, including the very low amounts of such glycoforms and their contamination with other forms of ECA (i.e., ECA\textsubscript{CYC} and ECA\textsubscript{PG}). The presented studies provide important insights that could prove helpful in further structural analyses and screening of ECA\textsubscript{LPS} among *Enterobacteriaceae* species.

Moreover, we are going to use a delipidated form of this antigen ([ECA]_n-dLOS) to prepare a neoglycoconjugate with tetanus toxoid to elicit antienterobacterial antibodies broadly cross-reactive with all forms of ECA and core OS epitopes. We hope to find them protective and a bactericidal solution against nosocomial infections of Gram-negative bacteria such as *Klebsiella, E. coli, Enterobacter, Proteus* spp., or *Serratia* spp. Since a small part of identified antigens were O-acetylated we are going to remove all O-acetyl groups from the [ECA]_n-dLOS preparation to produce a glycoconjugate vaccine that elicits the production of antibodies aimed at epitopes present in enterobacterial isolates with and without O-acetyl groups in their ECA, thus generating a wider-ranging response and higher serum bactericidal antibody titers. It is known that O-acetyl groups influence antigenicity and immunogenicity of poly- and oligosaccharide antigens.

**DEPARTMENT OF EXPERIMENTAL ONCOLOGY**

**Head: Professor Leon Strządała, Ph.D.**

**Laboratory of Experimental Anticancer Therapy**

**Head: Professor Joanna Wietrzyk, Ph.D.**

*Studies on the mechanisms of tumor progression, metastasis and on the effects of experimental antitumor therapy*

**Anticancer activity of tyrosine kinase inhibitor combined with docetaxel and vitamin D\textsubscript{3} derivative PRI-2191 in A549 non-small cell lung cancer model in vivo**

Tumor angiogenesis is one of the targets for anticancer therapy. Malignant cells, including lung cancer cells, release many growth factors that are involved in tumor vasculature formation. Blocking the signal transduction pathway of VEGF, the main angiogenic mediator, results in endothelial cell apoptosis, which in turn blocks angiogenesis. Endothelial cells appeared to be affected also by chemotherapy, initially designed to destroy cancer cells. In our study we decided to combine tyrosine kinase inhibitor (TKI) with VEGF receptor inhibitory activity, together with docetaxel as therapy in a non-small cell lung cancer (NSCLC) model *in vivo*. We also introduced a third compound, PRI-2191 vitamin D\textsubscript{3} analog,
in order to strengthen the effectiveness of the proposed therapy. Female NOD/SCID mice were injected s.c. with A549 cells and when the tumor volume reached approximately 80 mm³, the treatment schedule involving TKI, docetaxel and PRI-219 was applied. During the course of the experiment tumor size was measured and tumor growth inhibition was calculated. Mice receiving TKI alone or in combination with docetaxel and/or with PRI-219 had a smaller mean tumor volume compared to the control group, whereas the tumor volume in mice receiving TKI with docetaxel and/or with PRI-219 was statistically significantly smaller than in the docetaxel treated group. Combinations of TKI, docetaxel and PRI-219, tested \textit{in vitro} on HLMC endothelial cells, were more active in proliferation inhibition than each agent used alone and TKI combined with docetaxel.

Our findings suggest improvement of therapy consisting of TKI and docetaxel in NSCLC when applied with PRI-2191.

\textbf{Results of grant activities}

\textit{Anticancer activity of LPS complexes}

Lipopolysaccharide (LPS) can inhibit tumor growth in animal cancer models, which appears to be promising for cancer immunotherapy. However, its use in human cancer therapy has been limited to only one trial, since it is toxic, causing sepsis. Numerous peptides have been designed to bind and neutralize LPS. One of them is a peptide, polymyxin B, which prevents the occurrence of noxious LPS effects during LPS-mediated endotoxin shock in animal models. In order to obtain the anticancer effect of LPS while avoiding its toxicity, we investigated the influence of LPS complexes with polymyxin and LPS complexes with anti-LPS antibody on lung metastases formation in mice bearing B16 melanoma. In mice treated with both LPS complexes the number of metastatic foci was approximately 50-70\% lower in comparison to the control group of mice.

\textit{Naturally occurring isothiocyanates potentiate doxorubicin cytotoxicity in doxorubicin-resistant colon cancer cells}

Effective treatment of malignancies is a serious challenge since normally proliferating cells and cancer cells remain almost equally vulnerable to classical chemotherapy. As a result, high toxicity and severe side effects are often associated with cytostatic-based cancer treatment, limiting its applications. Moreover, chemotherapy very often leads to drug resistance, which further decreases the chance for successful treatment and full recovery. Thus, a new approach is desperately needed. One strategy is the concomitant use of a second compound which should act though cancer cell sensitization or reduction of chemotherapy-
related side effects, ultimately increasing treatment effectiveness. Isothiocyanates are among the most extensively studied natural compounds as potential anticancer drugs present in cruciferous vegetables in high amounts and variety. Numerous studies have shown that isothiocyanates such as benzyl isothiocyanate or sulforaphane are able to induce cell cycle arrest and apoptosis in a variety of cancer cell lines in vitro and in vivo. Recent studies proved their high potential as sensitizing agents in combined treatment with cisplatin, oxaliplatin, curcumin, 5-fluorouracil, metformin and others.

Our preliminary results indicated that naturally occurring isothiocyanates are able to reduce colon cancer cells’ resistance to doxorubicin viability in vitro, thus increasing the effectiveness of the treatment by exploiting several distinct mechanisms of action, ultimately resulting in an increased rate of apoptotic cell death. Naturally occurring isothiocyanates proved to be potent sensitizing agents when used along with the doxorubicin in a properly designed schedule. Pre-treatment with isothiocyanate gives a strong synergistic effect, whereas slight synergism or antagonism is observed after co-treatment. Their main mechanism of action in combined treatment appears to be based on modulation of the cell cycle and redox status partially correlated with glutathione level. Doxorubicin level remained at the same level after isothiocyanates pretreatment; therefore increased drug concentration appears not to be involved in increased cytotoxicity.

**Anticancer activity of 5-fluorouracil and its prodrug given with 1,4-dimethylpyridinium chloride and clopidogrel in murine colon cancer model**

The aim of the presented studies was to evaluate the therapeutic efficiency of 5-fluorouracil and capecitabine given simultaneously with clopidogrel and 1,4-dimethylpyridinium chloride to female B6 mice bearing murine colon cancer (MC-38/EGFP). Female B6 mice were subcutaneously inoculated with MC-38/EGFP cells. From the 1st day of the experiment mice were given 1,4-DMP (100 mg/kg/day, in drinking water). Administration of the 5-fluorouracil (35 mg/kg/day, q2d10), capecitabine (350 mg/kg/day, q1d14) and clopidogrel (10 mg/kg/day, continuously with drinking water) was started on the 27th day of the experiment. Mice were weighed and tumor volume measured three times each week. At the end of the experiment mice were euthanized, and tumor tissue, lymph nodes and blood samples were collected for further analysis. Results of the presented studies indicate that both 1,4-DMP and clopidogrel, when given alone, do not influence the growth of MC-38/EGFP tumor. Also, capecitabine in a proposed administration regime revealed a limited antitumor effect. 5-fluorouracil inhibited tumor growth by approximately 50%, and its tumor growth
inhibiting activity was additionally improved when the drug was simultaneously administered with 1,4-DMP (TGI value of 70%).

*Ex vivo* visualization of the lymph nodes indicated that all of the tested compounds influenced metastasis. Intensity of light emitted by the MC-38/EGFP cells localized in lymph nodes was three times lower in the groups treated with 1,4-DMP, clopidogrel or the combination of these compounds. Surprisingly, the highest metastasis inhibition was observed for the groups treated with capecitabine alone or in various combinations (more than 10-fold decrease in the emitted light intensity). Moderate antimetastatic activity was observed for 5-fluorouracil and its combinations. Additionally, we found that 1,4-DMP when given with cytostatic drugs decreased the blood level of alanine aminotransferase or aspartate aminotransferase, which may suggest its liver protective function during chemotherapy. 1,4-dimethylpyridinium chloride may beneficially influence the effects of selected cytostatic drugs, serving as an adjuvant agent enhancing anticancer activity and alleviating side effects induced by chemotherapy. The mechanism of its action remains to be established.

**Laboratory of Biomedical Chemistry**  
**Head: Professor Janusz Boratyński, Ph.D., Eng.**

The Laboratory of Biomedical Chemistry is part of The Laboratory of Experimental Oncology and Innovative Technologies “Neolek”. The laboratory is dedicated to research at the interface of chemistry and biochemistry. We create our own preparations; we have obtained hybrid macromolecules and we are excited with their biological properties. Our preparations act like a Trojan horse: they transport the drug to the target where the liberation of its activity occurs. Our laboratory collaborates with Polish industry: Adamed and Finepharm.

**Drug-carrier conjugate**

The subject of the invention is nanoparticles obtained as a result of the chemical linkage of two drugs: methotrexate (MTX) and hydroxyethyl starch (HES). Nanoparticles of increased antitumor activity compared to MTX were obtained. We described the chemical and physicochemical properties of the conjugate. Antileukemic activity was assayed in the Laboratory of Antitumor Experimental Therapy, part of “Neolek”. The antitumor effect *in vivo* was tested on mice burdened with the human leukemia cells MV4-11, which were subcutaneously implanted. The innovative preparation was administered intravenously once on day 6, when the average size of the tumor was $202 \text{ mm}^3$. 
During the therapy transient tumor disappearance was observed. For instance on day 18 in the group of non-treated mice the tumors had an average volume of 1409 mm$^3$, while in the group treated with the conjugate they were not detectable in 5 out of 7 mice, and their average volume was 50 mm$^3$ (in the group receiving free MTX it was 982 mm$^3$).

**Technology of bacteriophages – purification procedure**

Bacteriophages are viruses that invade bacterial cells, and, in the case of lytic phages, disrupt bacterial metabolism and cause bacterium to lyse. They can be used in the treatment of bacterial infections, as well as in biotechnology. The main contaminant of crude phage cultures on Gram-negative bacteria is lipopolysaccharide (LPS, endotoxin, pyrogen). LPS is a toxic molecule provoking a strong response from the immune system. That is why LPS removal is essential for preparations of bacteriophage required for experimental therapy. The method of endotoxin removal described for crude bacteriophage suspension uses extraction to the organic phase in the presence of magnesium ions and membrane filtration. The proposed method is scalable, fast and efficient. For example, bacteriophages cultured on *Escherichia coli* B containing 1950 EU/ml in lysate after a purification process contain only 4 EU/ml.

**Laboratory of Tumor Molecular Immunobiology**

**Head:** Professor Leon Strządała, Ph.D.

**Epigenetic silencing, oncogenic KRas and HIF-1 regulatory pathways in control of BNIP3 expression in human colorectal cancer cells**

Our studies focus on the impact of Ras oncogene, one of the most common alterations associated with human malignancy, on the expression of *BNIP3* in normoxic and hypoxic conditions. We describe the profound effect of KRas on the expression of *BNIP3* in colorectal cancer cells, where *BNIP3* expression was silenced by methylation, and explore the interplay between HIF-1 and the hypoxia pathway and oncogenic KRas in this context. We found that demethylation-triggered *BNIP3* expression (as observed in DLD-1 and HT-29 cells) remains uniquely dependent on the activity of Ras. We show that hypoxia or pharmacological activation of HIF-1 alone contributes to, but is not sufficient for, efficient induction of *BNIP3*. While *BNIP3* is a regulatory target of the hypoxia response pathway, the existence of KRas hyperactivity may be a necessary pre-condition for this effect to materialize in the molecular environment of human CRC cells. Additionally, in some experimental settings 5-aza-dC acted as a chemosensitizer. However, we demonstrate that 5-aza-2’-dC-mediated and Ras-
dependent BNIP3 expression results in resistance to 5-fluorouracil, a finding of potential clinical relevance.

_Thrombospondin-1 receptor mediates autophagy of RAS-expressing cancer cells and triggers tumor growth inhibition_

Thrombospondin-1 (TSP-1), a potent endogenous angiogenesis inhibitor, has attracted considerable attention due to its anticancer effects. This effect is largely attributed to the interaction between the endothelial CD36 receptor and the region of TSP-1 containing the type-1 repeats (TSR). Since the effects of these agents alone were less dramatic than those of full-length TSP-1, questions could be raised about the cancer-related roles of additional TSP-1 domains and receptors, such as CD47, which binds C-terminal sequences of this protein.

In a recent study we presented data showing the selective loss of viability of H-Ras-transformed cells upon ligation of the CD47 receptor. Interestingly, cells treated with anti-CD47 agents (4N1K peptide or anti-4N1K peptide CD47 antibody) did not exhibit hallmarks of classical apoptosis. On the other hand, an increase in staining with acridine orange and punctate staining for LC3 can be observed, both features reminiscent of autophagy. The 4N1K peptide administration also caused modest but specific and significant tumor growth inhibition in vivo.

Overall, our study offers a new element in the interactions between cancer cells and their surrounding matrix, including TSP-1. We propose that the loss of TSP-1 expression in H-Ras-transformed cells could bear an unappreciated significance (and selective advantage), as a way to remove the growth-restraining effects of TSP-1/CD47 interactions. These findings point to the role of pericellular and paracrine networks that may control oncogene function, oncogene addiction, therapeutic responses and tumor-stromal interactions of transformed cells.

DEPARTMENT OF IMMUNOLOGY OF INFECTIOUS DISEASES
Head: Professor Andrzej Gamian, Ph.D.

Laboratory of Medical Microbiology
Head: Professor Andrzej Gamian, Ph.D.

_Studies on the pathogenesis of some diseases of bacterial etiology and the role of bacterial surface glycoconjugates and protein antigens in the immune response_

The laboratory aims at the clarification of pathogenicity mechanisms of diseases with bacterial etiology, the role of bacterial glycoconjugates and proteins in the immune processes, the structure and functions of bacterial exopolysaccharides and endotoxins. Regarding the
studies on the relation of structure of surface antigens of probiotic exopolysaccharides and their role in immune system activation, experiments have been performed on a murine model of unspecific inflammatory bowel disease, using probiotic *Lactobacillus* strains. The structure of exopolysaccharide (EPS) has been established for *L. johnsonii* strain 151, isolated from healthy mice, and its serological reactivity compared with EPS of *L. johnsonii* strain 142 from colitis mice. It appeared that polysaccharides 151 and 142 of different structures had distinct activities. Polysaccharide 151 from healthy mice expressed broader serological activity than EPS 142 isolated from mice with colitis, when rabbit immune sera and human healthy blood donors were analyzed. This is a crucial observation for understanding the pathogenicity mechanisms of IBD. Studies of a specific peptide epitope present on enterobacterial OmpC protein which is recognized by umbilical cord antibodies revealed that this peptide conjugated with tetanus toxoid is immunogenic and induces in mice antibodies specific to OmpC. Such conjugates are good candidates for a vaccine. Continuation of studies on phage proteins allowed us to obtain two recombined *Klebsiella pneumoniae* phage KP32 proteins. Tubular tail proteins A and B appeared to have depolymerizing activity towards several polysaccharides. Studies on sialic acid containing O-specific polysaccharides from *Escherichia coli* O56 and O24 revealed that rabbit antibodies anti-O56 and anti-O24 polysaccharides cross-reacted with human tissue structures, especially in tumors. The epitope recognized by anti-O56 antigen is a specific marker of glandular epithelium, due to the fact that malignant glandular tumor and its metastasis are stained, and also epithelium of renal tubules and glandular structures of the thyroid gland are stained. The most relevant observation is that the epitope recognized by anti-O56 antibodies is a new marker specific for glandular epithelium and nervous tissue.

**Laboratory of Virology**  
**Head: Professor Egbert Piasecki, Ph.D.**  
**Study on nonspecific immunity in viral infections**

About 8% of the human genome consists of endogenous retroviral sequences originating from germ cell infections by exogenous retroviruses during evolution. Most of those sequences are inactive because of accumulation of mutations but some of them are still capable of being transcribed and translated. The latter include insertionally polymorphic HERV-K113 and HERV-K115. It has been suggested that their presence and expression are connected with several human diseases. It is also believed that they could interfere with the replication cycle of exogenous retroviruses, including HIV. Prevalence of endogenous
retroviral sequences HERV-K113 and HERV-K115 was determined in the Polish population. The frequencies were determined as 11.8% for HERV-K113 and 7.92% for HERV-K115. To verify the hypothesis that the presence of these HERV sequences could affect susceptibility to HIV infection, comparison of a control group (HIV-negative, not exposed to HIV; n = 303) with HIV-positive patients (n = 470) and exposed but uninfected (EU) individuals (n = 121) was performed. Prevalence of HERV-K113 and HERV-K115 in the EU group was 8.26% and 5.71%, respectively. In the HIV(+) group we detected HERV-K113 sequences in 12.98% of the individuals and HERV-K115 sequences in 7.23% of the individuals. There were no statistically significant differences between groups studied. The frequency of HERV-K113 and HERV-K115 sequences in Poland was found to be higher than usually observed for European populations. No relation between presence of the HERVs and HIV infection was detected. The results were published in PLoS One, 2013; 8: e77820.

Vascular and metabolic dysfunctions and mitochondrial failure are now believed to be contributors to Alzheimer’s disease (AD) pathogenesis. Vascular dysfunction includes reduced cerebral blood flow (CBF), blood-brain barrier (BBB) disturbances and cerebral amyloid angiopathy (CAA). Mitochondrial failure results in deregulation of $\text{Ca}^{2+}$ homeostasis and elevated reactive oxygen species (ROS) generation, both of which are linked to neurotoxicity. Increased levels of ROS stimulate proinflammatory gene transcription and release of cytokines, such as IL-1, IL-6, and TNF-α, and chemokines, thereby inducing neuroinflammation. Conversely, inflammatory reactions activate microglia and astrocytes to generate large amounts of ROS, so neuroinflammation could be perceived as a cause and a consequence of chronic oxidative stress. The interaction between oxidative stress and neuroinflammation leads to amyloid-β (Aβ) generation. The deposition of Aβ peptide in the brain generates a cascade of pathological events, including the formation of neurofibrillary tangles (NFTs), inflammatory reactions, increased oxidative stress and mitochondrial dysfunction, which are causative factors of cell death and dementia. The purpose of this paper is to provide current evidence on vascular dysfunction and mitochondrial failure, both in neurons and glia and in brain vascular wall cells in the context of potential application for treatment of AD and other neurodegenerations. The results were published in CNS & Neurological Disorders – Drug Targets, 2013; 12: 870-881.
Elicitation of myelopoiesis in mice by homologous recombinant lactoferrin

Effects of lactoferrin (LF) on myelopoiesis have been a matter of controversy. One of the obstacles was unavailability of homologous LF. In this study we demonstrated that intravenous injection of mouse recombinant LF elicited dose-dependent (optimal dose of 100 μg) output of cells of a myelocytic lineage into circulation. Mouse transferrin, used as a control, was without effect. The increase in output of neutrophil precursors, neutrophils and eosinophils was correlated with a twofold increase of leukocyte levels. The analysis of the bone marrow sections confirmed increased myelopoiesis. The increased myelopoiesis was accompanied by elevated serum concentrations of interleukin 6 at 6 h and haptoglobin at 24 h following administration of LF. In conclusion, the homologous LF elicits significant and transient myelopoiesis in experimental mice.

Immunoregulatory actions of lactoferrin on proliferation of human nasal fibroblasts

Effects of human, recombinant lactoferrins (LFs) (obtained in HEK and CHO cells) and milk-derived LF and of fibroblast growth factor 2 (FGF-2) on fibroblast proliferation were studied. Milk LF had no effect on fibroblast proliferation but both recombinant LFs stimulated proliferation. FGF-2 had an enhancing effect on fibroblast proliferation stimulated by recombinant LFs. In addition, U0126, a highly effective inhibitor of MEK1 and MEK2, abolished LF-induced proliferation.

Immunoregulatory actions of human recombinant lactoferrin on cytokine production by blood cells from intensive care unit (ICU) patients

The studies were conducted on patients admitted to the ICU after postsurgical complications resulting in SIRS or septic shock. Blood samples were taken at three time intervals (days 1, 3 and 5 after admission). Whole blood cultures were stimulated with LPS, recombinant human LF (50 μg/ml) or both agents. The following cytokines were measured in the cell cultures: TNFα, IL-6, IL-8, IL-10 and IFNγ. Healthy volunteers constituted a control group. A regulatory effect of LF was observed with regard to pro-inflammatory cytokine production, in particular in patients who exhibited profound hyporeactivity upon admission to the ICU. The studies are in progress.
**Studies on immunoregulatory properties of cyclic peptides**

From a group of linear and cyclic peptides which were studied *in vitro* in 2012, the two most active compounds, that were devoid of toxicity (KJ3-6 and KJ3-3c), were selected for *in vivo* investigations. The compounds, administered intraperitoneally, were tested in the model of delayed type hypersensitivity (DTH) to ovalbumin and in the carrageenan foot pad test in mice. In the DTH test both compounds were strongly suppressive, both in the induction and effector phases. Similar, strong inhibitory actions of the compounds were observed in the carrageenan test. KJ3-3c (cyclic peptide) was more active and displayed suppressive action comparable to that of dexamethasone, a reference drug.

**Effects of plant preparations on cytokine production and activation of NF-κB in human endothelial cells and whole blood cell cultures**

The aim of this project was to evaluate the effects of several plant preparations on TNF-α and IL-6 production by human endothelial cell lines and whole blood cultures as well as to determine activation of NF-κB transcriptional factor. Mutagenic action was evaluated by the Ames test to exclude mutagenic extracts for further research and non-toxic concentrations of the preparations were also established. The studied preparations induced, in a dose-dependent manner, low production of the cytokines correlated with the activation rate of NF-κB. The obtained results are necessary to continue studies in other models.

**The influence of nickel on pig tissues**

Twelve animals were exposed to nickel ions released from a metal plate located on the inner side of their cheeks. The proliferation rates of porcine blood mononuclear cells obtained from control and metal plate-bearing animals were similar. Earlier studies performed on a smaller group of animals showed 50% increase of the proliferation rate of mononuclear cells from pigs with a nickel plate. A possibility exists that the higher number of animals in the experimental group increases tolerance to the nickel plate in the tested group or increases stress in the control group.

Metallothionein (MTn) is a protein found in two isoforms. MTn1 isoform is constitutively present in the body and is responsible for the removal of undesirable divalent metal ions, which may interfere with biological processes of the body. An excess of such ions could induce expression of the MTn2 isoform of this protein. Comparative studies of gene expression of both MTn isoforms performed in three tissues (lung, liver, and nerves) revealed the inclusion of a usually dormant gene in the liver of these animals.
Demonstration of the biological activity of BMP synthesized by the HeLa cell line as well as chondrocytes and fibroblasts

Chondrocytes, fibroblasts and HeLa cells have an ability to synthesize BMP. They also have receptors for these proteins. The biological activity of BMP is manifested by phosphorylation of a small signaling molecule, Smad. Furthermore, there is an increase of alkaline phosphatase activity (APL) as a result of activation of the ALP gene by SmadP. Such experiments have been preliminarily conducted, but without the use of blocking antibodies against the BMP-receptor reaction. Currently, in vitro studies of inhibition of the binding of BMP to the receptor are being conducted for the HeLa cell line, using specific ant-BMP-2/4 antibodies and competitive receptor activation with synthetic peptides, having the sequence of the BMP receptor binding domain. The expected results were masked by factors present in serum added to the culture medium despite lowering its concentration. The reaction observed in the control culture of HeLa competes with that of the culture treated with BMP. At present, searching for experimental conditions enabling correct detection of BMP is in progress.

Laboratory of Immunopathology
Head: Professor Irena Frydecka, M.D, Ph.D.

Studies on the mechanisms of immune deficiency in neoplastic and autoimmunological diseases

The research activity of the Laboratory of Immunopathology is mainly focused on scientific problems associated with the immune abnormalities attributed to neoplastic and autoimmune diseases.

Correlation of peripheral blood Th1, Th17, and Treg cell distribution with rheumatoid arthritis activity

In the pathogenesis of rheumatoid arthritis (RA), a special role is attributed to the local as well as systemic immune dysregulation. It has been shown that active RA results from an imbalance in the distribution of pro-inflammatory Th17 and anti-inflammatory Treg cells, which emphasizes the crucial roles of these cells in controlling RA. Recently, an affected Th1 response has been suggested to shift the T cell homeostasis from a Treg-mediated tolerant state to Th17-mediated active inflammatory conditions. Thus Th1/Th17/Treg subsets can act as important participants of the complex network of interactions that manage the development and progression of RA, and, what is more important, they can exhibit this action at different stages of the disease with different intensities. The aim of our study was to perform phenotypic and quantitative analyses of the proportions of PB CD4+ T cell subpopulations.
involved in RA pathogenesis, including Th17, Treg, and Th1 cells, in the context of disease activity evaluated according to the DAS28 score, to verify whether RA severity corresponds with the imbalance in circulating Th1, Th17, and Treg cell distribution. Forty-eight patients with active RA were enrolled in the study: 7 with LDA (low disease activity; DAS28 2.7-3.2), 18 with MDA (medium disease activity; DAS28 3.3-5.1), and 23 with HDA (high disease activity; DAS28>5.1). Phenotypic analysis was performed using flow cytometry, and particular cell subpopulations were defined as follows: Th1 (CD4+IL-17-IFN-gamma+), Th17 (CD4+IFN-gamma-IL-17+), and Treg (CD4+CD25++FoxP3+) cells. The values were expressed as the median percentages (IQ ranges). We observed a positive correlation between clinical and biochemical markers of inflammation (DAS28 and C-reactive protein (CRP) levels, respectively): the highest values of CRP were seen in the HDA group, intermediate levels in the MDA group, and the lowest levels in the LDA group. Although the Th1 population was found to be decreased in the HDA group, the differences were not statistically significant compared to the other groups of patients. Similarly, proportions of Treg cells were comparable between the groups examined. The only population of CD4+T cells dysregulated in PB was Th17, strongly enriched in the HDA group; there were no statistically significant differences in the percentages of PB Th17 cells between patients with MDA and LDA of RA. In addition, we demonstrated that proportions of Th17 cells in PB of active RA patients were positively correlated with both DAS28 and CRP values. Our study clearly shows that the magnitude of the Th17 population is the best systemic immune marker of RA severity.

An association of the genetic polymorphisms within genes encoding CD28, CTLA-4, and BTLA molecules with neoplastic diseases

Since T cells play a key role in antitumor immunity, abnormal expression of co-stimulatory molecules CTLA-4 and CD28 that regulate T-cell activity, and the newly discovered molecule BTLA (B and T lymphocyte attenuator), engaged in immune suppression, could influence cancer susceptibility. Abnormal expression may be caused by polymorphisms in genes encoding those molecules. Our laboratory has studied the association of genetic polymorphisms within genes encoding two members of the CD28-B7 superfamily, CD28 and CTLA-4, with renal cancer and prostate cancer. Two studied SNPs in the CTLA-4 gene, c.49A>G in exon 1 and g.319C>T in the promoter region, significantly increased the risk of prostate cancer but not renal cancer. The presence of adenine in exon 1 and thymine in the promoter region significantly increases the risk of prostate cancer (p= 0.009 and p= 0.047, respectively). Overall analysis of two other studied SNPs, CT60 and Jo31, in the 3’UTR
region of the CTLA-4 gene did not show any association of these SNPs with renal cancer or prostate cancer. However, in a group of patients with clear cell renal cell carcinoma (CCRCC) there was a trend toward lower frequency of CT60[AA] and Jo31[TT] genotype than in the control group ($p=0.055$, $p=0.065$, respectively). In the case of SNPs within CD28 we observed that women with the CD28c.-1042G>A[G] allele and CD28c.-1042G>A[GG] genotype significantly more often develop CCRCC but there was no association between two SNPs in the CD28 gene, c.17+3T>C and g.54779795G>A, and renal cancer in the overall population, or with prostate cancer. The third molecule in relation to which SNPs were studied in our laboratory was BTLA, an inhibitory molecule. The SNPs selected for analysis were studied to determine the relationship with risk of renal cancer (SNPs: rs2705535, rs9288952, rs1844089, rs9288953, rs1982809, rs2705511), prostate cancer (SNPs: rs9288952, rs2705535, rs2705535) and chronic lymphocytic leukemia (CLL, SNPs: rs9288952, rs1844089, rs2705535 and 9288953). The presence of rs9288953[T] allele in intron 1 of the BTLA gene significantly decreased the risk of CLL as compared to [CC] genotype ($p=0.002$). The haplotype rs9288952A>G[AA] /rs1844089G>A[G] /rs2705535C>T[C]/9288953C>T[C] significantly decreased the risk of disease ($p_{after Bonferroni correction}=0.01$) and the haplotype rs9288952A>G[AA] /rs1844089G>A[G]/rs2705535C>T[C]/9288953C>T[T] increased the risk of disease ($p_{after Bonferroni correction}=0.005$). There was no association with other studied SNPs with CLL or with renal and prostate cancer. Moreover, the SNP rs16859633 was not polymorphic in our population.

**Laboratory of Reproductive Immunology**

**Head: Professor Anna Chelmońska-Soyta, Ph.D., V.D.**

*Immunological mechanisms associated with reproductive processes in health and disease*

The research activity of the laboratory was mainly devoted to elucidation of embryo-maternal interactions in the local (uterus) and peripheral (spleen) compartment during the pre-implantation period of pregnancy in mice. Described studies are a continuation of studies performed last year.

Embryo signals in preimplantation pregnancy are important for establishment of the “split” tolerance mechanism which protects the semiallogenic embryo from immune recognition by the mother immune system and on the other hand maintains an appropriate level of mother immune response to infectious agents. Embryo signals are primarily detected locally in the uterus but very early signs of embryo presence are visible in peripheral organs of the maternal immune system. This year we showed that the proteome map of spleen CD4
lymphocytes is different in pseudopregnant and pregnant mice. CD1 mice were housed in a dark:light cycle (12h:12h) under SPF conditions. Mice were superovulated and females of the control group were mated with vasectomized males while mice from the experimental group were mated with males with proven fertility. At 3.5 days after mating mice were euthanized and uteri were checked for the presence of embryos. Spleens were dissected and CD4 lymphocytes were magnetically sorted. 2D image analysis and protein identification by mass spectrometry were performed. Twenty-five protein spots were significantly altered during the preimplantation period of pregnancy. Among them 13 were up-regulated and 12 were down regulated. Among the proteins that showed altered expression six of them (cofilin-1, GDR1 and GDR2, F-actin capping protein subunit alpha and beta and tropomyosin alpha) are key proteins involved in regulation of cell migration and immune synapse formation. This part of the research was done in collaboration with the Department of Physiology, Faculty of Biotechnology and Animal Breeding, West Pomeranian University of Technology in Szczecin.

In the local compartment (uterus) gene expression analysis revealed that most of the signal transduction pathways analyzed by Mouse Signal Transduction PathwayFinder PCR Array (www.sabiosciences.com) are down-regulated, particularly the WNT, LDL and estrogen pathway. We showed that the observed increased COX-2 expression (one of 7 genes which were up regulated) is concomitant with PPARgamma level down-regulation both at mRNA and protein level.

In conclusion: The obtained results suggest very precise selection of locally induced signals which may regulate the peripheral response to the presence of the embryo before i

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

Laboratory of Molecular Biology of Microorganisms
Head: Professor Jolanta Zakrzewska-Czerwińska, Ph.D.

Replication and segregation of bacterial chromosomes
Polyketide synthesis and its regulation in Streptomyces

The research activity of the Laboratory of Molecular Biology of Microorganisms (LMBM) is mainly focused on two scientific problems: i/ replication and segregation of bacterial chromosomes, and ii/ polyketide synthesis and its regulation in Streptomyces.

i/ DNA replication is an important event of the bacterial cell cycle. The decision to initiate DNA replication is crucial for the fate of the cells, and depends both on the
intracellular as well as on the environmental signals. In LMBM we are interested in mechanisms of the initiation and regulation of bacterial chromosome replication, with special emphasis on characterization of the key factors engaged in initiation complex formation, namely the initiator protein (DnaA) and the origin of chromosome replication (oriC) as well as their reciprocal interactions leading to formation of the nucleoprotein complex (orisome) able to unwind the DNA double helix. We also aim at the identification and functional analysis of regulators which control replication and coordinate the initiation with the cell cycle progression. We are especially focused on orisome formation in the pathogenic epsilonproteobacterium *Helicobacter pylori*, in which the bipartite oriC1-oriC2 origin structure has been discovered. The DnaA binding sites at oriC1 were previously characterized, but no DnaA boxes were identified at oriC2. Recent analyses allowed us to map DnaA-binding sites at oriC2; their function in DNA unwinding and interactions with the DnaA-oriC1 subcomplex is currently being investigated. It is also interesting to analyze whether some features of orisome formation, thus far unique for *H. pylori*, are also important for orisome assembly in related bacteria. Thus a new project has been started, which aims at identification of oriC regions and analyses of orisome formation in selected bacteria from the epsilon class of proteobacteria, which include bacteria leading different lifestyles: pathogenic, host associated and non-pathogenic, free living species. These studies should allow us to propose the initiation mode of the chromosomes of the epsilonbacteria as well as indicate the common and unique features for the whole class. Additionally we started to characterize key elements (e.g. oriC and DnaA) responsible for initiation and regulation of *Bdellovibrio bacteriovorus*. *B. bacteriovorus* is a small Gram-negative predatory bacterium that invades other larger Gram-negative bacteria and exhibits a fascinating life cycle comprising two major stages: a non-replicative stage (outside its prey) and a replicative stage inside its prey. In addition to characterization of *B. bacteriovorus* orisome, our research might help to discover how *Bdellovibrio* switches between axenic and predatory growth.

Replication and segregation of bacterial chromosomes are interconnected: DNA is progressively segregated as the sister chromosomes are synthesized. In bacteria, the accurate segregation of the newly replicated chromosomes is facilitated by ParA and ParB proteins. ParA forms filamentous structures, which enable transport of segregation complexes toward cell poles. In LMBM, we study segregation of chromosomes in *Streptomyces coelicolor* and *Mycobacterium smegmatis*. We have shown that ParA interacts with the pole associated proteins such as DivIVA in *M. smegmatis* and Scy in *S. coelicolor*. DivIVA is a polar growth determinant, while Scy is a component of the tip-organizing centre that controls tip growth.
The interaction of the segregation machinery with pole determinants may suggest that bacterial ParA homologues are associated with cell division and/or cell elongation. Our studies will be continued by comparative analysis of the heterologous ParA interactions with its partner proteins using the *E. coli* system.

ii/ Bacteria from the genus *Streptomyces* are potent producers of polyketides – a large class of bioactive compounds with extremely diverse structures and functions. They are synthesized as secondary metabolites by giant multienzyme complexes – polyketide synthases. The secondary metabolism of streptomycetes is launched in concert with morphological differentiation and the regulatory network which governs these processes is best studied in the model organism *Streptomyces coelicolor* A3(2). Our work is focused on a polyketide synthase, Cpk from *S. coelicolor* A3(2). Cpk is responsible for the synthesis of a yellow pigment, coelimycin. Expression of *cpk* genes is tightly controlled. We aim at identification of the regulatory pathway controlling expression of *cpk* genes and synthesis of coelimycin. There are five regulatory proteins encoded by the genes within the *cpk* cluster and several pleiotropic regulators which are suggested to be involved in regulation of *cpk* expression. In our studies we proved that one of the proteins from the cluster is an activator of Cpk synthase expression and it is subjected to feedback control. We also found that one of the pleiotropic regulators represses the synthesis of coelimycin and actinorhodin (another polyketide of *S. coelicolor* A3(2)).

**Laboratory of Signaling Proteins**

**Acting Head: Professor Janusz Matuszyk, Ph.D.**

*Studies on proteins and signaling pathways involved in activation of proinflammatory transcription factors and response to hypoxia*

Nucleoside adenosine acts as a regulatory molecule and induces the response to hypoxia, including biosynthesis and release of catecholamines from the cells of the adrenal medulla. Adenosine induces expression of the tyrosine hydroxylase (TH) gene encoding the rate-limiting enzyme in the synthesis of catecholamines. However, it is suggested that atrial natriuretic peptide (ANP) inhibits expression of the TH gene. Using real-time PCR and luciferase reporter assays we found that ANP significantly decreases the adenosine-induced transcription of the TH gene in rat pheochromocytoma PC12 cells. Results of measurements of cyclic nucleotide concentrations indicated that ANP-induced accumulation of cyclic GMP (cGMP) inhibits the adenosine-induced increase in cyclic AMP (cAMP) level. Using selective phosphodiesterase 2 (PDE2) inhibitors and a synthetic cGMP analog activating PDE2, we found that PDE2 is involved in coupling the ANP-triggered signal to the cAMP metabolism.
We have established that ANP-induced elevated levels of cGMP as well as cGMP analog stimulate hydrolytic activity of PDE2, leading to inhibition of adenosine-induced transcription of the TH gene. We conclude that ANP mediates negative regulation of TH gene expression via stimulation of PDE2-dependent cAMP breakdown in PC12 cells.

DEPARTMENT OF CLINICAL IMMUNOLOGY

Laboratory of Clinical Immunology
Head: Professor Andrzej Lange, M.D.

The continuous effort of the laboratory focuses on transplantation immunology. In the last year we concluded our observation based on the immunogenetic data collected from 8 institutions involved in hematopoietic stem cell transplantation and donor–recipient matching. The novelty of our research is documented by the following findings:

1. Functional Single Nucleotide Polymorphism of the IL-10 gene has already been shown as associated with acute graft-versus-host disease (aGvHD) incidence risk post-transplant. In our study we documented that this functional polymorphism affects differentiation of naïve T cells into T regulatory cells, which in turn makes patients more or less susceptible to alloreactivity.

2. The NOD2/CARD15 gene encodes the cytosol receptor recognizing muramyl dipeptide residues present in bacterial walls. Three polymorphic features affecting the function of this receptor were analyzed and for the first time we showed that each of these polymorphisms specifically affects different signal transduction pathways, which results in susceptibility to (1) sepsis (lack of proper surveillance of bacterial infection associated with enhanced potential of cytokine generation) in SNP 13 carriers and (2) Herpes viruses reactivation and aGvHD in SNP8 positive patients.

3. The novel technique of intra-bone (IB) – directly to leukemic lesions – infusion of donor lymphocytes (DLI) was developed. These infusions were clinically effective with the regression of leukemic lesions. Our study on lymphocyte infiltration compositions post IB DLI documented the local expansion of activated CD8+ cytotoxic cells. Intra-bone lesion infusion of T cells facilitates cell-to-cell contact between leukemic cells and lymphocytes, which results in local expansion of cytotoxic cells with anti-leukemic potential.

4. Our study on lymphocyte infiltration composition in chronic graft-versus-host disease (cGvHD) lesions documented the presence of IL-17 producing cells in the digestive tract but not in the skin. The extent of lymphocyte/IL-17 producing cells infiltration correlated well with the painful symptomatology. Also it was shown that in the digestive tract
lymphoplasmacytoid cells may be IL-17+. The observation showed that the distribution of IL-17-producing cells in the cGvHD pathological lesions recapitulates that in the physiological situation in man.

5. A follow-up study of patients post allo-HSCT documented that the number of T regulatory cells at the hematological recovery influences the final outcome of transplantation. Patients with a higher number of those cells enjoy longer survival but those with lower numbers suffer more frequently from aGvHD and herpes virus reactivation. The observation shows that the very first confrontation between the host cells and those transplanted affects the late outcome of the procedure.

6. The study on the presence of endothelial stem cells in the marrow created the rationale behind revascularization attempts in patients with ischemic legs and avascular necrosis of the femur head, which appeared to be successful. These successful studies developed the competence of our group, which was very helpful in organizing a conference on “Biological and clinical aspects of the use of somatic stem cells in regenerative medicine” (Wroclaw, December 16th-17th, 2013). In this conference we had an outstanding faculty and 130 participants from 11 countries (www.conference2013.dctk.wroc.pl).

Editorial activity of the year 2013 was also effective. Promoted by the previous year’s successful publication of a Special Issue entitled “Biological and Genetic Aspects of Donor-Recipient Matching in HSCT” published in Bone Marrow Research which had more than 33000 visits and 3300 downloads) we completed the publication of a manual entitled “Manual of immunogenic diagnostic procedures involved in donor-recipient matching for hematopoietic stem cell transplantation”.

Laboratory of Immunogenetics and Tissue Immunology
Head: Professor Piotr Kuśnierczyk, Ph.D.

Immunogenetics of human diseases

Killer cell immunoglobulin-like receptor (KIR) gene association with atopic dermatitis (AD)

AD is a common skin disease of complex etiology including affected humoral and cellular immune responses. The role of NK cells in development of this disease has been recently postulated, but is still poorly documented. The current study was undertaken to determine the impact of genes for the most polymorphic NK cell receptors, known as KIRs, on the development of AD.

We compared 240 patients suffering from AD with 570 healthy controls. Frequencies of the great majority of KIR genes did not differ between patients and controls, except for KIR2DS1,
whose frequency was significantly (OR = 0.629, CI95% (0.45; 0.87), $p_{corr} = 0.0454$) lower in patients than in controls. These results were confirmed in a second cohort of 201 patients. When both patient groups were combined and compared to the control group, the result for KIR2DS1 achieved even higher significance (OR = 0.658, CI95% (0.5; 0.86), $p_{corr} = 0.0158$).

To the best of our knowledge, this is the first report on KIR gene contribution to AD, and to allergy in general [Niepiekło-Miniewska et al., Gene, 2013].

This work was done in collaboration with the Department of Dermatology, Venereology and Allergology, Wrocław Medical University, Wrocław, Poland, with the 1st Department of Dermatology and Venereology, Medical University of Łódź, Łódź, Poland, with the 2nd Chair of Internal Diseases, Medical University of Łódź and N. Barlicki Medical University Hospital, Łódź, Poland, with the Department of Medical Genetics and the Department of Prevention of Environmental Hazards and Allergology, Medical University of Warsaw, Warsaw, Poland, and with the City of Hope Comprehensive Cancer Center, Duarte, California, USA.

**Distribution of KIR genes in global populations**

In the last fifteen years, published reports have described KIR gene content frequency distributions in more than 120 populations worldwide. However, there have been limited studies examining these data in aggregate to detect overall patterns of variation at regional and global levels. With our data for the Polish population, we contributed to a description of the collection of KIR gene content data for 105 worldwide populations, collected as part of the 15th and 16th International Histocompatibility and Immunogenetics Workshops [Hollenbach et al., Int. J. Immunogenet., 2013].

**ALCAM, a novel locus for susceptibility to multiple sclerosis, interfering with HLA-DRB1*1501**

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system mediated by an autoimmune process. Activated leukocyte cell adhesion molecule (ALCAM) is a molecule involved in leukocyte migration across the blood–brain barrier, which is a key stage in MS pathogenesis. The present study is the first to report evidence of the association of rs6437585 ALCAM gene polymorphism with risk and progression of MS. Our investigation revealed that rs6437585CT individuals had higher risk of MS (OR = 2.34; 95%CI = 1.22–4.51; $P = 0.011$) and over 2 years earlier age of onset (95%CI = 0.16–4.41, $P = 0.036$).
The *HLA-DRB1*1501 allele was reproducibly found to be the most strongly associated with susceptibility to MS, and this was also confirmed in our study. However, we demonstrated that two ALCAM polymorphisms modify this effect. We found that two ALCAM polymorphisms, rs11559013 and rs34926152, although not associated with MS itself, modify *HLA-DRB1*1501 effect. Results obtained from logistic regression analysis showed five-fold lower risk for MS for both rs11559013GA/*HLA-DRB1*1501+ and rs34926152GT/*HLA-DRB1*1501+ individuals. These observations may suggest a protective role against MS for both rs11559013GA and rs34926152GT genotypes in *HLA-DRB1*1501 positive individuals [Wagner et al., J. Neurol., 2013].

This work was done in collaboration with the Department and Clinic of Neurology, Medical University, Wroclaw, Poland.

**Association of LILRA3/ILT6 deletion variant with later onset of multiple sclerosis**

Recently published studies have implicated the deletion polymorphism in the LILRA3 gene as being associated with multiple sclerosis (MS). A total of 309 patients diagnosed with MS and 379 unrelated healthy volunteers were typed for 6.7-kbp deletion in the LILRA3 gene. Simultaneously, presence or absence of the *HLA-DRB1*1501 allele was established to assess the possibility of interaction between LILRA3 deletion and *HLA-DRB1*1501 status. Similarly to most other European populations we found significantly higher frequency of the *HLA-DRB1*1501 allele in cases than we found in controls (27.0% vs. 12.5%; p < 0.0001, OR = 2.6, 95%CI = 1.96–3.42). However, in contrast to previous reports, we did not find any association of LILRA3 deletion with MS susceptibility. Also, the *HLA-DRB1*1501 stratification analysis showed no LILRA3 association with the disease. However, we observed that patients negative for the deletion may begin to suffer from MS significantly earlier than patients who are positive (p = 0.014). [Wiśniewski et al., Hum. Immunol., 2013].

This work was done in collaboration with the Department and Clinic of Neurology, Wroclaw Medical University, Wroclaw, Poland.

**Laboratory of Clinical Immunogenetics and Pharmacogenetics**

**Head:** Professor Katarzyna Bogunia-Kubik, Ph.D.

**VEGF and bFGF gene polymorphism in patients with hematological disorders (chronic lymphocytic leukemia and non-Hodgkin’s lymphomas)**

Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) play an important role in the initiation of angiogenesis. Both VEGF and bFGF have been reported to have prognostic significance in non-Hodgkin’s lymphomas (NHL) and B-cell
chronic lymphocytic leukemia (B-CLL). The objective of the present study was to assess whether polymorphisms located within the genes coding for these key angiogenic activators (VEGF (rs3025039; C>T) and bFGF (rs308395, G>C)) contribute to disease susceptibility and/or progression in patients with hematological disorders (B-CLL and NHL).

A slight prevalence of the VEGF T variant was observed among patients as compared to healthy individuals (p=0.095) with a significant difference when high risk (stage III/IV) patients were considered (OR=3.81, p=0.045).

The presence of the VEGF T allele was found to significantly associate with worse prognosis of NHL (expressed by the highest International Prognostic Index; IPI). The bFGF G variant was more frequently detected among patients with aggressive as compared to those with indolent histological subtype and healthy individuals (OR=2.51, p=0.038).

These results suggest the association of the VEGF polymorphism with high risk B-CLL and imply that VEGF and bFGF gene polymorphisms have prognostic significance in patients with NHL. They were published in 2013 in *Med Oncol* and *Biomed Res Int* journals.

**CXCL12 and CXCR4 gene polymorphism in patients with multiple myeloma**

Multiple myeloma (MM) is a plasma cell malignancy characterized by bone marrow infiltration and the presence of a monoclonal protein in serum and/or urine. CXCR4 and its ligand CXCL12 are essential for neoplastic cell homing to bone marrow in hematological malignancies. The present study aimed to determine the association between the polymorphic features located within the CXCR4 (rs2228014, A>G) and CXCL12 (rs1801157, C>T) genes and disease susceptibility and progression.

The CXCR4 T allele was less frequently represented among patients (OR=0.074, p<0.001). The CXCL12 gene polymorphism was found to affect the course of the disease. The CXCL12-3’A variant was significantly more frequently present in patients with less advanced MM according to stages of the disease as well as the International Staging System (ISS) score. The favorable effect of the CXCL12-3’A allele was also seen in the analysis of patient survival.

The data were published in 2013 in the journal *Cytokine*.

**The impact of the selected single nucleotide polymorphisms on the efficacy of therapy with TNF-α inhibitors in patients with rheumatoid arthritis**

Despite the fact that therapy with TNF-α inhibitors constitutes a breakthrough in rheumatoid arthritis management, no improvement is still achieved in approximately 30% of cases. Our study aimed to evaluate whether the TNFA promoter (rs1800629, G>A; rs3615525, G>A; rs1799724, C>T), TNFRI A (rs767455, G>A) and TNFRI B (rs1061622, T>G); HLA-E
(rs1264457, C>T; Arg128Gly) and NKG2A receptor (rs7301582 C>T; rs2734440 A>G) polymorphisms affect the efficacy of therapy with TNF-α inhibitors in patients with rheumatoid arthritis (RA) who have been treated for at least 3 months or they stop therapy because of adverse events. A moderate EULAR response (according to the European League Against Rheumatism criteria) was achieved in 69% of patients, while a good EULAR response was achieved in 23% of patients at 3 months. At week 24 low disease activity or remission was achieved by 45% of the patients. It appeared that homozygosity for the TNFR1A A allele could act as a genetic factor associated with better response to anti-TNF treatment while a significantly worse response (DAS28>5.1) was observed in patients carrying the HLA-E C allele, NKG2A-(rs7301582)-CC genotype or NKG2A-(rs2734440)-AA genotype. Also treatment failure (inefficiency or loss of effectiveness of therapy) was more frequently observed in the HLA-E CC homozygous patients as well as in those with the NKG2A-(rs2734440)-AA genotype.

These results imply that the polymorphisms within genes coding for non-classical HLA-E and its NKG2A receptor, as well as the receptor for TNF-alpha, affect the response to therapy with TNF inhibitors in patients with RA.

**DEPARTMENT OF TUMOR IMMUNOLOGY**

**Head: Professor Pawel Kisielow, Ph.D.**

**Laboratory of Molecular and Cellular Immunology**

**Head: Professor Malgorzata Cebrat, Ph.D.**

_Ikaros and RAG-2-mediated antisense transcription are responsible for inactivation of the promoter of the NWC gene in lymphocytes_

Expression of NWC, the third evolutionarily conserved gene in RAG (RAG-1 and RAG-2 locus), is regulated by a promoter localized in the RAG-2 intron. The promoter is inactive in immature and mature lymphocytes. We have previously shown that the inactivation of the NWC promoter is caused by methylation of a CpG island which is localized within the 5’-end of the gene. The aim of our study was to identify factors responsible for lymphocyte-specific methylation of the NWC promoter. We found that the NWC promoter contains two consensus binding sites for Ikaros (lymphocyte-specific transcription factor) which is known to act, depending on the context, either as a transcriptional activator or a repressor. Ikaros binding sites in the NWC promoter overlap with binding sites for ZFP-143 transcription factor, which we found to be the promoter activator. We therefore hypothesized that lymphocyte-specific expression of Ikaros leads to the
replacement of ZFP-143 in the NWC promoter, which results in promoter inactivation and its subsequent methylation. We have shown that:

- Ikaros binds to both putative binding sites in the NWC promoter and that the binding is non-cooperative.
- Overexpression of Ikaros in non-lymphoid cells results in downregulation of NWC gene expression.
- Overexpression of Ikaros results in downregulation of NWC promoter activity which proves that the direct binding of Ikaros to the promoter is responsible for downregulation of NWC gene expression.
- Ikaros is able to outcompete ZFP-143 from their mutual binding, which is a plausible explanation for the mechanism of Ikaros-dependent downregulation of NWC promoter activity.
- Overexpression of Ikaros in non-lymphoid cells does not result in methylation of the NWC promoter.

Based on these results we further hypothesized that the lymphoid-specific methylation of the NWC promoter can be caused by expression of the RAG-2 transcript, which is both antisense towards NWC expression and lymphoid-specific. To verify this idea we constructed a transgenic mouse model containing a full-length RAG/NWC locus obtained from a bacterial artificial chromosome (modified to create RAG-2/GFP and NWC/YFP fusion genes) and a transcriptional termination cassette inserted immediately downstream of the first exon of the RAG-2 gene. The offspring of two transgenic founders was analyzed to assess the DNA methylation level at the NWC promoter in developing and mature lymphocytes. We found that the methylation level of the NWC promoter, when compared to control transgenic mice (without the termination cassette) was downregulated by 50% to 80% in lymphoid cells and was not affected in non-lymphoid cells, proving that the antisense transcription is a cis-acting factor responsible for methylation of the NWC promoter in lymphoid cells.

We concluded that both Ikaros and antisense RAG-2 transcription are responsible for NWC promoter inactivation in lymphocytes. A plausible mechanism is that Ikaros expression in early lymphocyte precursors is responsible for at least transient downregulation of NWC promoter activity enabling transcription of the RAG-2 gene which could be otherwise hindered by NWC transcription due to transcriptional interference. RAG-2 transcription in turn would cause methylation of the NWC promoter and its permanent inactivation, for which Ikaros availability and binding to the promoter would be dispensable.
Laboratory of Tumor Immunology  
Head: Professor Arkadiusz Miążek, Ph.D.  

*Degradation of the linker for activation of T cells (LAT): molecular switch of T cell function*

The research in the Laboratory of Tumor Immunology focuses on elucidating a role of altered T cell receptor (TCR) signaling in development and progression of acute T cell leukemia/lymphoma. For this we have generated a transgenic mouse model in which 100% of animals that are deficient in the transmembrane adapter protein LAT, a major TCR signaling node, develop aggressive thymic lymphoma by 6-8 weeks of age. These mice are being used to delineate the earliest molecular pathways involved in tumor formation and progression. We have isolated a minute population of thymocytes whose cell surface phenotype indicates early pre-leukemic changes associated with suboptimal pre-TCR signal transduction. We aim at characterizing gene expression signatures of this pre-leukemic stage and finding out if this phenotype can be reversed by ectopic expression of a missing TCR signaling component.

To extend our studies beyond murine models we examined the sensitivity of human leukemic T cell lines, in which the expression of LAT was strongly downregulated by shRNA knock-down, to pro-apoptotic drugs. We found that the LAT adapter transmits essential tumor pro-survival signals whose diminution leads to increased sensitivity to pro-apoptotic drugs. We are now testing several strategies to decrease the expression of LAT in tumor T cell lines in order to find out if LAT can be a target for anti-tumor therapies.

LABORATORY OF GLYCOBIOLOGY AND CELLULAR INTERACTIONS  
Head: Professor Danuta Duś, Ph.D.

*Mechanisms of tumor progression. Intercellular adhesive interactions during metastatic spread of cancer cells  
Biology of endothelial progenitor cells*

**Aim:** Our main aim was to prepare model endothelial cell lines, representing different endothelial cell differentiation stages. These model cell lines will be applied for studies on the regenerative potential of endothelium, especially in regard to vascular endothelial damage in some diseases, such as myocardial infarction. On the other hand, they may also allow for elaboration of a tumor neoangiogenesis inhibition strategy.

**Research done:** Experiments were designed using two established cell lines of human early endothelial progenitor cells (HEPC-CB.1 and HEPC-CB.2). Preliminary data were obtained using the protein array technique where the secretion profile of these cells was measured. This
method allows for simultaneous measurement of semiquantitative production of different cytokines. Using a commercially available protein membrane, the profile of cytokines produced by both endothelial progenitor cell lines in normoxia and hypoxia was determined. According to the results obtained from the protein matrix, the cells of both lines secrete both pro-angiogenic (MCSF, GRO, CXCL16, MCP-3, PlGF, Ang, VEGF, IL-7, IGFBP-2, MCP-1, IL-8) and anti-angiogenic factors (TIMP-1, TIMP-2). It was also demonstrated that hypoxia induces increased secretion of almost all pro-angiogenic factors by both cell lines tested (apart from IGFBP-2).

**Conclusion:** It has been shown that the endothelial progenitor cell lines are capable of supporting angiogenesis of mature endothelial cells via secretion of various factors.

**Use of the results:** The study may allow one to better understand the biology of endothelial progenitor cells, and to determine their regenerative potential, allowing the possible use of these cells in the treatment of diseases in which damage of the endothelial layer is present.

**Galactosylceramide affects tumorigenic and metastatic properties of breast cancer cells as an anti-apoptotic molecule**

It was recently proposed that UDP-galactosylceramide galactosyltransferase (UGT8), an enzyme responsible for synthesis of galactosylceramide (GalCer), is a significant index of tumor aggressiveness and a potential marker for the prognostic evaluation of lung metastases in breast cancer. To further reveal the role of UGT8 and GalCer in breast cancer progression, the tumorigenicity and metastatic potential of control MDA-MB-231 cells (MDA/LUC) and MDA-MB-231 cells (MDA/LUC-shUGT8) with highly decreased expression of UGT8 and GalCer after stable expression of shRNA directed against UGT8 mRNA were studied in vivo in athymic nu/nu mice. Control MDA/LUC cells formed tumors and metastatic colonies much more efficiently in comparison to MDA/LUC-shUGT8 cells with suppressed synthesis of GalCer after their, respectively, orthotopic and intracardiac transplantation. These findings indicate that UGT8 and GalCer have a profound effect on tumorigenic and metastatic properties of breast cancer cells. It was also found that expression of UGT8 in MDA-MB-231 cells increased their resistance to apoptosis induced by doxorubicin in vitro. Therefore, these data suggest that accumulation of GalCer in tumor cells inhibits apoptosis, which would facilitate metastatic cells to survive in the hostile microenvironment of the tumor in the target organ.
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