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Research Report 2012

LABORATORY OF BACTERIOPHAGES
Head: Professor Andrzej Górski, M.D.

The presence of bacteriophages in alimentary tract of patients with inflammatory bowel diseases

Determination of frequencies and concentrations of bacteriophages for strains: own strain *E. coli* from examined humans, *E. coli B*, *E. coli 1962* and *E. coli DSM 13127* in the stools of healthy volunteers and patients with inflammatory bowel diseases (IBD) (Crohn's disease and colitis ulcerosa) was studied. The correlation between the number of *E. coli* bacteria and frequencies of coliphage occurrence and number of coliphages in the intestine of patients and volunteers was also analyzed. Stools of 20 healthy volunteers, 24 patients with Crohn's disease and 30 with colitis ulcerosa were examined. The material from adult patients was obtained from the Clinic of Gastroenterology and Hepatology of the Academic Clinical Hospital in Wrocław.

Frequencies of coliphage occurrence in feces of patients were lower (5.5%-51.8%) compared with healthy persons (15%-70%). The concentrations of coliphages in stools were greater in patients (10^3-10^8 PFU/g) than in volunteers (10^1-10^6 PFU/g). The frequencies of isolation of *E. coli* bacteria were lower in stools of patients with IBD (87%) than in volunteers (100%). The mean concentration of *E. coli* bacteria was greater in patients' stools ($8.1x10^7$ CFU/g) than in volunteers ($1.2x10^7$ CFU/g).

A correlation was observed between number of *E. coli* bacteria and coliphage frequencies and concentrations in stools of volunteers and patients with IBD. Under the bacteria concentration 10^6 CFU/g there were lower frequencies and concentrations of coliphages in human stools. Between the bacteria concentration 10^6 and 10^9 CFU/g there were higher frequencies and concentrations of coliphages. The results were statistically insignificant.

Neutralization of phages by sera of patients with bacterial infections undergoing phage therapy

The antiphage sera activity in patients with bacterial infections treated with phages at the Phage Therapy Unit at the Institute of Immunology and Experimental Therapy and sera from healthy volunteers were examined. The antiphage sera activity of 83 patients treated with the phages *S. aureus, E. coli, P. aeruginosa*, *K. pneumoniae*, *E. faecalis*, *S. marcescens* and *M. morganii* was determined. The phages were administered orally, locally or intrarectally.

The sera of 30 healthy volunteers neutralized *E. coli T4* phage, and *S. aureus* phages *676/Ż* and *A3/R* up to a serum dilution of 1:100. Weak activity of sera was detected in patients prior to phage administration up to a serum dilution of 1:100. Higher activity of sera diluted above 1:800 was observed in 18.1% of examined patients treated with phages from 7 to 60 days. Higher antiphage activity was found in patients with *S. aureus*, *E. faecalis* and *P. aeruginosa* infections undergoing phage therapy. Mostly higher antiphage sera activity was observed in patients using phages locally. Patients using phages orally had low antiphage sera activity.

Characterizing the biology of novel lytic bacteriophages infecting multidrug resistant Klebsiella pneumoniae

Klebsiella spp. are one of the leading microbial pathogens associated with nosocomial infection. Increase in the incidence of antimicrobial resistant *Klebsiella* species has propelled the need for alternate/combination therapeutic regimens to aid clinical treatment. Bacteriophage therapy forms one of these alternative strategies. This study characterizes the lytic/therapeutic potential of 32 *Klebsiella* phages isolated from sewage and environmental water samples.

Of 32 isolated phages 8 belonged to the *Myoviridae*, 8 to the *Siphoviridae* family whilst the remaining 16 belonged to the short tailed *Podoviridae* family. The lytic potential of these phages was characterized against 254 clinical *Enterobacteriaceae* strains including multidrug resistant *Klebsiella* isolates producing extended-spectrum beta-lactamases (ESBLs). Based on the therapeutic/lytic potential, six of the 32 phages were further characterized for lytic potential, physicochemical properties and phage genome restriction endonuclease patterns. Through restriction endonuclease digestion of the phage genomes they were found to be genetically different. Interestingly, through downstream genomic analysis of these six phages multiple phage-encoded host resistance mechanisms were identified. The *Siphoviridae* phage

genomes (KP16 and KP36) were targeted by both Dam and Dcm DNA modification by host methyltransferases but also encode low numbers of host restriction modification sites similar to the evolved strategy of T7-like phages. The φKMV-like KP34 phage was sensitive to all endonucleases used in this study. Dam methylation of KP34 DNA was detected although this was in the absence of an identifiable phage encoded methyltransferase gene. The *Myoviridae* phages KP15 and KP27 were both identified to carry phage-encoded Dam and Dcm methyltransferase genes and other anti-restriction mechanisms elucidated in previous studies. No other anti-restriction mechanisms were detected, e.g. atypical nucleotides (hmC or glucosyl hmC) although *Myoviridae* phage KP27 encodes an unknown anti-restriction mechanism that needs further investigation.

Immunogenicity studies of T4 phage head proteins

T4-like phages are multi-antigenic objects, ubiquitous in the biosphere. Thus, anti-T4-like antibodies are probably frequent in mammals. One of the important issues that contribute to the success or failure of therapeutic use of bacterial viruses is the immunogenicity of phages, since antibodies affect phage effectiveness in the body. Further, new attempts to apply T4 phage capsids as platforms for effective presentation of antigens in vaccines add to the importance of phage immunogenicity. However, the contribution of particular capsid proteins to the antigenic reactivity of phage particles is unspecified. Here we present comparative studies of immunogenic properties of the T4 phage head surface proteins gp 23*, gp 24*, gp Hoc and gp Soc in reference to antibody induction.

Heterologous expression of the proteins in *E. coli* with specific chaperones and purification by affinity chromatography and size-exclusion chromatography were applied. Two-step lipopolysaccharide removal allowed immunological purity grade. Native conformations of the proteins were confirmed using circular dichroism. Whole bacteriophages were purified similarly from liquid cultures. Antibody production was studied in sera of mice injected with phages/proteins (immunization) or healthy human volunteers (natural antibodies) by ELISA immunoassay.

Briefly, in the mouse model of immunization, the primary response (IgM) was induced mostly by gp 23* (Major Capsid Protein) and gp soc then gp hoc, and the least immunogenic was gp 24*. Immunogenicity of phage proteins located on complete phage capsids was different to individual immunogenicity of isolated proteins: specific IgM was effectively stimulated only by gp hoc. In the secondary response (IgG) two proteins were highly immunogenic, with a comparable high level of antibodies: gp 23* and gp hoc. The other two,

gp 24* and gp soc, were almost ineffective in IgG induction. Lack of gp hoc (T4Δ*hoc*) did not affect immunogenicity of other surface T4 head proteins. In human healthy volunteers (50 individuals), significantly elevated levels of IgG antibodies only against gp 23* were found.

Interestingly, one of the T4 head proteins which was named highly immunogenic (gpHoc), and which is often proposed as the most effective stimulator of the immunological response, was found not to be the most immunogenic in general. The most immunogenic was Major Capsid Protein (gp 23*), which forms the main surface of the T4 phage head.

The most important results of grant activities

The results of treatment of 153 patients admitted for phage therapy (PT) done within the protocol *"Experimental phage therapy of drug-resistant bacterial infections, including MRSA infections"* at the Phage Therapy Unit (PTU) between January 2008 and December 2010 were summarized. The efficacy of treatment was evaluated based on the results of bacterial cultures, the assessment of the intensity of infection symptoms, the results of specific diagnostic tests, and the opinions of consulting medical specialists. Overall, a good response to PT was found in 39.9% of patients while pathogen eradication and/or recovery defined as complete subsidence of the infection symptoms was noted in 18.3% of cases. The highest percentage of positive outcomes (48.3%) as well as pathogen eradication and/or recovery was found in the group of men with urogenital infections (37.9%). The phage preparations were essentially well tolerated by 77.8% of patients and only in 3.9% of the analyzed subjects was PT terminated due to adverse reactions. PT did not impair the function of the bone marrow as well as such vital organs as kidneys, liver and pancreas, although in some patients deviations from the norm were noted for isolated parameters (they had transient character or were not clinically significant and did not require special treatment). Interestingly, the analysis of the changes in the phage typing profile of the major bacterial strains isolated from the patients revealed that bacterial resistance to the specific therapeutic phage occurred in 17.0% of *Staphylococcus*, 36.4% of *Pseudomonas*, 42.9% of *E. faecalis*, and 86% of *E. coli* strains. In general, these data suggest that PT can provide good clinical results in patients with otherwise untreatable chronic bacterial infections and is essentially well tolerated. Although they have an observational character, they may be of significance for the development of clinical trials aimed at introducing PT as a standard therapy.

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

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

In order to obtain the native CEA N-A1 domains, the proper length of the A1 domain sequence must be determined first. We used for this purpose several bioinformatic tools and the directed mutagenesis technique. Several constructs with different C-terminals were created and tested. The best results were obtained using N-A1 domains with Asn-185 as a C-terminal. We have also elaborated the optimal fusion tag, expression conditions and the protein purification procedure. The most important point was the purification procedure because the domains were present in the solutions as soluble aggregates composed of N-A1 domains, MBP-N-A1 fusion fragment and cleaved MBP. In order to isolate pure N-A1 domains, we had to disrupt aggregates. To achieve that, we tested several chemical agents. The best results were obtained using 1.25 M guanidine chloride, which caused aggregate dissociation but did not denature the domains. We also characterized the biochemical properties of the domain using several techniques, for example analytical gel filtration and circular dichroism, which revealed that obtained N-A1 domains are homogeneous and possess the proper structure. Analytical gel filtration also indicated that the domains are present in the solution as dimers, so they can be used in the determination of amino acids responsible for the homotypic interactions. In order to find out those amino acids two mutated N-A1 domain constructs were prepared using the directed mutagenesis technique – one construct with Val-39 substituted by Ala and the second containing a double mutation: Val-39 into Ala and Asp-94 into Ala.

Rare polyagglutinable NOR erythrocytes contain three unique globoside (Gb4Cer) derivatives, NOR1, NOR_{int} and NOR2, in which Galα1-4-, GalNAcβ1-3Galα1-4- and Galα1-4GalNAcβ1-3Galα1-4, respectively, are linked to the terminal βGalNAc residue of Gb4Cer. NOR1 and NOR2, which both terminate with the Galα1-4GalNAcβ1- sequence, react with anti-NOR antibodies, commonly present in human sera. While searching for an enzyme responsible for biosynthesis of the Galα1-4GalNAcβ1- sequence, we identified a C631G mutation in the *A4GALT* gene encoding Gb3/CD77 synthase (α1,4-galactosyltransferase). We showed that the C631G mutation alters the acceptor specificity of Gb3/CD77 synthase, rendering it able to catalyze synthesis of both Galα1-4Gal- and Galα1-4GalNAc- moieties. In

order to estimate the frequency of NOR phenotype in the Polish population, 464 non-related individuals were evaluated using anti-NOR monoclonal antibody and TaqMan Genotyping Assay. It was found that all individuals were NOR-negative and were homozygous for C at position 631. Five NOR-negative individuals were homozygous for C according to TaqMan Genotyping Assay, but sequencing of the open reading frame revealed CC homozygosity. It seems that the false positive results are caused by copy number variation.

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

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

Proline-rich polypeptide complex (PRP) with immunoregulatory and antioxidative properties showed a positive clinical effect in the case of Alzheimer's disease (AD) especially when administered to patients in the early stages of the disease. Some of PRP's complex properties are reflected by its nonapeptide constituent (NP). In the case of neurodegenerative diseases, including AD, intensification of oxygen reactive species (ROS) and nitric oxide (NO) release is observed. High reactive nitrogen species (RNS) created from ROS and NO can modify the protein through tyrosine nitrification. An endogenously created neurotoxin, nitrotyrosine (NT), plays a role in cell destruction, and its elevated level is observed in neurodegenerative diseases, including AD. So, we decided to check the effect of PRP and its constituent peptide, NP, on protein nitrification using human blood cell leukocytes and cell line J774 as a model of microglia cells in the central nervous system and PC 12 cell line. In cell lysates obtained from cells cultured without and with the oxidative stress inducer LPS, in the presence or absence of peptides, NT levels were measured with commercially available ELISA tests. No statistically significant effect of PRP and NP was observed.

The results obtained indicate that the modulatory effect on nitrosylation of proteins induced by increased release of NO is not an element of positive effects of PRP/Colostrinin.

Results of grant activities

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

The influence of proline-rich polypeptide (PRP) and its nonapeptide constituent on the signaling pathway nNOS-cGMP-ERK 1/2 was described in PC12 cells. NGF treatment resulted in neurite outgrowth observed in at least 80% of cells. Treating cells with PRP (at a dose of 0.1 μg/ml for 5 days) induced neuritis extension in 30% of cells. No effects were observed for higher doses of PRP. After treatment with NP (0.1 μg/ml) only 5% of cells

undergo differentiation. With use of the RT-PCR technique 100x elevated expression of nNOS was observed, which was in accordance with the level and activity of the protein observed using Western blotting technique and in confocal microscopy (monoclonal anti-nNOS antibodies). In the presence of PRP and NP increased secretion of NO was observed in 77% and 95%, respectively, which positively correlated with the effect on cyclic guanyl cyclase activity. Specific effects of NOS and sGC inhibitors indicate that neuritogenesis is connected with nNOS/NO/sGC/cGMP.

It was shown that in the presence of PRP and NP activity of kinase ERK 1/2 was significantly higher. This effect was abolished in the presence of specific inhibitor of MEK kinase (U0126) activating ERK 1/2 kinases.

In preliminary experiments the influence of PRP and NP on cell viability in the presence of toxic doses of amyloid β was shown as well as modulation of nNOS expression, intracellular NO level and cGMP induced by Aβ.

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

In 2012, the fourth step of the grant project was performed. The aim of this study was the creation of hFcRn promoter-reporter constructs: wild-type containing hFcRn promoter fragment –660 to +52; mutant reporter constructs containing promoter fragment –660 to +52 with substitutions in the binding motifs for the transcription factors. The promoter fragment –660 to +52 was chosen, because within this fragment there were identified previously sequences participating in specific DNA-protein interactions, and transcription factors binding to these sequences.

A 711 bp fragment (containing nucleotides –660 to +52) of the hFcRn promoter was subcloned into the KpnI/NheI sites of the promoterless firefly luciferase reporter expression vector pGL3-Basic to generate the pGL3-Basic/711 reporter construct of the wild type. The resulting expression plasmid was verified by restriction enzyme digestion and the orientation of the insert was confirmed by DNA sequencing. This construct possesses promoter activity in the chosen cell lines THP1, Caco-2, HUVEC, Lu 106.

The mutant promoter constructs were generated using the Quickchange Lighting Site-directed Mutagenesis Kit. The pGL3-Basic/711 construct served as the template for the site-directed mutagenesis. The promoter reporter constructs were purified using “Plasmid Midi AX Kit” and all mutations were confirmed by DNA sequencing. Fourteen mutant promoter constructs were created. Each individual mutant promoter construct possesses the mutated one consensus

binding site. Because within a 711 bp fragment of the hFcRn promoter there were identified three binding sites for the transcription factor Sp1 family, two for the nuclear protein CF1/YY1 family, and two for the C/EBP family, there were also generated mutant promoter constructs from which each individual possesses all mutated binding sites for a given transcription factor. All promoter reporter constructs will be used in the studies to evaluate the contribution of the identified binding sites within the hFcRn promoter, and transcription factors specifically binding to these sequences in the transcriptional regulation of the human *hFcRn* gene.

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

Many of the mechanisms of innate immunity depend on fast, flexible and straightforward interactions between soluble or cell-associated pattern-recognition molecules (PRMs) and pathogen-associated molecular patterns (PAMPs) – conservative structures common for a variety of pathogens. Examples of such PRM–PAMP are the collagen-associated lectins such as ficolin-1 (M), ficolin-2 (L), ficolin-3 (H, Hakata antigen) and mannan-binding lectin (MBL), together with their microbial ligands. These molecules are involved in complement activation, via the lectin pathway – an important element of the innate immune response. This unique feature depends on the ability of these lectins to form complexes with MBL-associated serine proteases. The C-terminal fibrinogen-like region of ficolins constitutes the target-recognizing domain.

Identification of ligands for ficolins is a prerequisite to understand the role of the collagen-related lectins in innate immunity and to design diagnostic tools to measure concentration and the activity of these key complement components in body fluids, e.g. serum. Ficolin-3 is an oligomer of subunits consisting of three identical polypeptide chains. Despite similar molecular organization, biochemical properties and activities, it differs from other proteins of that family as the amino acid sequence homology between ficolin-3 and ficolin-1 or ficolin-2 is 48%. Ficolin-3 is synthesized in the liver by hepatocytes and bile duct epithelial cells and in lungs by type II alveolar and ciliated bronchial epithelial cells. It is secreted into blood, bile ducts, bronchi and alveoli and therefore it may participate in both systemic and local innate immune responses. The average concentration of ficolin-3 in human sera is 18 μg/mL, the highest among ficolins and significantly exceeding the MBL level.

The importance of serum ficolin-3 was indicated by several reports concerning disease associations with abnormally low or high concentrations. Lower levels of ficolin-3 were observed among patients suffering from systemic lupus erythematosus and sarcoidosis. A reduced ficolin-3 level in hepatic cirrhosis may be a marker of impaired liver function. An enhanced risk of febrile neutropenia (especially with bacteremia) in ficolin-3-deficient pediatric cancer patients treated with chemotherapy as well as necrotizing enterocolitis among neonates was demonstrated recently.

The specificity of ficolin-3 is poorly characterized and currently limited to a few ligands only. We present new specific targets for human ficolin-3, identified among lipopolysaccharides (LPSs, endotoxin) of *Hafnia alvei*. The interaction was limited to LPSs of four strains: 23, Polish Collection of Microorganisms (PCM) 1200, PCM 1203 and PCM 1205 and limited to their O-specific polysaccharides (O-specific PSs) composed of different numbers of oligosaccharide (OS) repeating units (RUs). These LPS/ficolin-3 complexes activated the lectin pathway of complement in a C4b-deposition assay in a calcium- and magnesium-dependent way.

A neoglycoconjugate of the O-specific PS fraction of *H. alvei* 1200 LPS with bovine serum albumin (BSA) was prepared and used as a tool for the determination of ficolin-3 and activity and concentration in serum. To confirm the structure of the O-specific PS 1200 selected for the conjugate preparation, structural analysis was performed on a series of O-specific PSs released by the mild acid hydrolysis of the LPS. The isolated O-specific PSs, showing the different length distributions, were devoid of a major part of the core OS region and had Hep-Kdo disaccharide at the reducing end.

The neoglycoconjugate was a highly selective tool for the determination of ficolin-3 activity and concentration in serum, which is not affected by MBL, ficolin-1 and ficolin-2 or natural antibodies.

DEPARTMENT OF EXPERIMENTAL ONCOLOGY
Head: Professor Leon Strządała, Ph.D.

Laboratory of Experimental Anticancer Therapy
Head: Professor Joanna Wietrzyk, Ph.D.

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

Combination of endothelium-directed drugs and cyclophosphamide – a comprehensive approach to cancer treatment

The ability of cancer cells to detach from the primary tumor, then to migrate through the vascular system and finally to colonize distant tissues and form drug-resistant and irremovable lesions still remains the leading cause of death among cancer patients. Therefore a therapeutic approach based on a combined treatment directed against the tumor growth as well as the invasive potential of cancer cells may be a promising strategy for a comprehensive anticancer therapy.

Pyridinium salts such as 1-methylnicotinamide chloride (MNA) and its derivative 1,4-dimethylnicotinamide chloride (1,4-DMP) were previously shown to influence the metastatic process when given both alone as well as in a combination with cytostatic or antiplatelet drugs. Such observations encouraged us to further investigate their activity in triple drug combinations.

In our recent study carried out in orthotopically implanted 4T1 mouse mammary gland cancer cells we showed that 1,4-DMP exceeds the activity of MNA when salts are given in a combination with cyclophosphamide and the antiplatelet drug clopidogrel. 1,4-DMP not only preserved anticancer activity of the applied cytostatic (tumor growth inhibition remained at the level of 40-55% from the 15th until the last day of the experiment); moreover it also decreased in 80% the number of lung metastases when compared to the control group of animals ($P<0.05$) and in 50% in comparison to the group treated with cyclophosphamide. It is also worth noting that we did not observe any symptoms of systemic toxicity while the treatment was carried out.

Our further efforts devoted to the optimization of pyridinium salts dosage schedule resulted in the conclusion that the tested agents show antimetastatic activity when applied before cancer cells initiate their migration through the vascular system. This consequently convinced us that MNA and 1,4- DMP may be effective agents in cancer metastasis prevention.

Our studies confirmed that pyridinium salts given as an adjuvant may improve the efficiency of standard chemotherapy and if given early enough may prevent the development of metastases. In our opinion such observations support the idea of a complex anticancer treatment based on the modulation of vascular and endothelial activity.

Interaction of endotoxin and polymyxin B in B16 mouse melanoma model of metastasis

Lipopolysaccharides (LPS) have been recognized as an efficient immune stimulator as well as multicytokine inducer and they are believed to have potent antitumor and antimetastatic activity through a host-defense mechanism. So far it has been demonstrated that

LPS could regress tumor growth in animal cancer models, which is promising even for cancer immunotherapy in humans. However, its use in human cancer therapy has been limited only to one trial, since it is toxic, causing sepsis.

Numerous peptides have been designed to bind and neutralize LPS. One of them is a natural peptide, polymyxin B (PMB), which prevents noxious LPS effects occurrence during LPS-mediated endotoxin shock in animal models. The polymyxin B mechanism of action depends on its interactions with bacterial cell membrane phospholipids, which results in disruption of membrane structure.

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 metastasis formation in mice bearing B16 melanoma. In mice treated with LPS and polymyxin the number of metastatic foci was approximately 50-70% lower in comparison to the control group of mice. We did not observe an antimetastatic effect in mice treated with LPS alone. Treatment with polymyxin alone showed stimulation of metastatic foci formation.

Laboratory of Biomedical Chemistry
Head: Professor Janusz Boratyński, Ph.D., Eng.
Studies on drug-carrier conjugates

The Laboratory of Biomedical Chemistry is a branch of the Integrated Laboratory of Experimental Oncology & Innovative Technologies "Neolek".

Research focuses on two major fields: drug-carrier systems, and bacteriophages. Carried small molecules include therapeutic agents such as methotrexate (1), and carboranes (2,3) with potential in boron neutron capture therapy (BNCT). Proteins and oligosaccharides are employed as the carrier molecules. Promising results were obtained for methotrexate-hydroxyethyl starch (HES) conjugate, which led to a significant decrease of tumor size in advanced stage experimental MV-4 leukemia after administration of a single dose (4). Biological experiments are performed within the united Neolek laboratory.

The bacteriophage research comprises physiochemical studies and development of process technologies. One of the developed methods was a procedure for purification that diminished levels of endotoxins by several orders of magnitude. The laboratory is equipped with modern HPLC hyphened with MS/MS and DLS. We are open for cooperation with small and large companies and other research groups.

The laboratory is applying for the GLP certificate.

(1) Biochim Biophys Acta. 2012, 1830(3):2526-2530. doi: 10.1016/j.bbagen.2012.11.005.

(2) patent pending 398379 PL

(3) patent pending 398380 PL

(4) patent pending 398253 PL

(5) patent 213388 PL

Laboratory of Tumor Molecular Immunobiology
Head: Professor Leon Strządała, Ph.D.
Molecular mechanisms of cell death induced in human leukemia/lymphoma cell lines by fenretinide in combination with indomethacin

Clinical trials with different pharmacological inhibitors in cancer therapy show that the use of these compounds alone is not very effective in fighting cancer. Therefore, an alternative solution in targeted therapy is to apply a combination of drugs triggering different mechanisms of action. Combination therapy has a more effective impact on many physiological processes (apoptosis, differentiation, angiogenesis and metastasis), allowing us to use much lower concentrations of the compounds, and reduces the possibility of resistance by cancer cells. Therefore in our investigations we examined the effects of fenretinide [N-(4-hydroxyphenyl)-retinamide] in combination with the non-steroidal anti-inflammatory drug (NSAID) indomethacin (INDO). We provide evidence that combination treatment with fenretinide and INDO results in Jurkat cell death, which is peculiar and differs from classical apoptosis. Our observations suggest that induced death is not accompanied by activation of effector caspase-3 but may go through a different AIF-mediated pathway that is considered as caspase-independent. In detail we observed: cell membrane permeabilization, phosphatidylserine exposure, lack of oligonucleosomal DNA fragmentation, lack of caspase-3 activation, but apoptosis inducing factor (AIF) nuclear translocation and cleavage of the nuclear protein PARP. According to some literature reports, treatment with drugs targeting only apoptotic pathways does not often provide a sufficient approach to cure cancer. What is important, combination of fenretinide and indomethacin was nontoxic for human peripheral blood lymphocytes (PBL).

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 is involved in studies of pathogenicity mechanisms of diseases with bacterial etiology, the role of bacterial glycoconjugates and proteins in the immune processes, and the structure and functions of bacterial exopolysaccharides and endotoxins. Regarding the studies on the method of determination of markers specific for Gram-negative and Gram-positive bacteria, its usefulness was proved for serum samples of patients with sepsis and septic shock. The method may serve as an additional tool in a diagnostic laboratory for clinical practice. Concerning the difficult problem of identification of actinomycetal clinical isolates, experiments revealed that apart from glycolipids and mycolic acids, also exopolysaccharides are relevant as useful markers which facilitate diagnostic procedures due to better specificity of these antigens. In order to understand the serological specificity, structural studies have been initiated on exopolysaccharides produced by several actinomycetal strains. Studies on probiotics are focused on the relation of structure and functions of their exopolysaccharides. Polysaccharide of one of the probiotic *Lactobacillus paracasei* strains appeared to have immunomodulating properties when murine bone marrow cells were studied. Polysaccharides of different structures had distinct activities. One of these antigens markedly diminished IL-12 synthesis and increased the IL-10 level, which prompted us to study further the basis of this phenomenon. The other project concerns studies on enterobacterial OmpC protein, which is recognized by umbilical cord antibodies. The specific peptide epitope present in this protein has been identified and synthesized. Also the amino acid chemical functions crucial for activity in this epitope were identified. Conjugates with tetanus toxoid based on this peptide were obtained and their beneficial properties were defined as candidates for investigation towards a vaccine. The conjugate is devoid of undesirable epitopes which are present in isolated OmpC protein.

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

Effects of chemokine receptor alleles (*CCR5-Δ32* and *CCR2-64I*) on susceptibility to human immunodeficiency virus (HIV) infection were studied in a Polish population. The *CCR5* and *CCR2* genotypes were determined for 311 healthy, HIV-negative individuals (control group), 121 exposed to HIV infection but uninfected (EU group), and 470 HIV-positive patients. The frequency of the alleles in the control group was calculated as 0.12 for both *CCR5-Δ32* and *CCR2-64I*. The logistic regression method was used to analyze the effects of the described factors. A protective effect was observed for the *CCR5-Δ32* allele but

only in the case of heterosexual exposure. Prevalence of the *CCR5-Δ32/+* genotype in HIV^+ patients infected via the heterosexual route (n = 61; 8.2%) was much lower than in the control group (n = 311; 21.5%); in the heterosexually exposed uninfected group it was slightly higher (n = 28; 25%). This suggested that in this mode of infection, the native CCR5 expression level was crucial for establishment of infection. Individuals with the *CCR5-Δ32* allele have more than three times less chance of infection in the case of HIV heterosexual exposure (odds ratio, 3.37; 95% confidence interval, 1.055–10.76). However, a protective effect of the *CCR5-Δ32/+* genotype was not observed in the case of intravenous drug users (IDUs). The frequencies of the genotype were similar in HIV-infected IDU individuals (n = 356; 17.7%) and in exposed uninfected patients (n = 84; 15.5%), not significantly different from the control group. No effect of the *CCR2* genotype was observed. The analysis revealed that the important factor increasing infection risk was, in particular, hepatitis C virus (HCV) infection (odds ratio, 12.9). Moreover, the effect of HCV infection was found to be age dependent. Susceptibility to HIV infection resulting from HCV positivity became weaker (6% per year) with increasing age. The results were published in *AIDS Research and Human Retroviruses 2013; 29: 54-60.*

Alzheimer's disease (AD) is a debilitating illness with no known cure. Nowadays accumulating evidence suggests that the vascular endothelium and chronic hypoperfusion may play an important role in pathobiology of AD. The vascular endothelium, which regulates the passage of macromolecules and circulating cells from blood to tissue, is a major target of oxidative stress, playing a critical role in the pathophysiology of vascular diseases. Since the vascular endothelium, neurons and glia are all able to synthesize, store and release reactive oxygen species (ROS) and vascular active substances in response to certain stimuli, their contribution to the pathophysiology of AD can be very important. New evidence indicates that continuous formation of free ROS induces cellular damage and decreases antioxidant defenses. Specifically, oxidative stress increases vascular endothelial permeability and promotes leukocyte adhesion. We summarize the reports that sporadic, late onset of AD results from vascular etiology. Recently the involvement of epigenetic alterations in the etiology of AD has also been intensively investigated. Gaining a more complete understanding of the essential components and underlying mechanisms involved in epigenetic regulation could lead to novel treatments for a number of neurological and psychiatric conditions. The results were published in *Journal of the Neurological Sciences 2012; 323: 25-32.*

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

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

Effects of lactoferrin on elicitation of the antigen-specific cellular and humoral cutaneous response in mice

Immune contact dermatitis is an inflammation of the skin resulting from exposure to allergens in the environment. We compared the actions of bovine lactoferrin (LF), a natural immunomodulator, on the elicitation phases of the cellular and humoral, cutaneous immune responses to oxazolone and toluene diisocyanate (TDI), respectively. LF was given i.v. in a 10 mg/mouse dose, together with the eliciting doses of the antigens. The ear edema and the number of lymphocytes in the draining lymph nodes were measured. In addition, the production of IL-2 in the cultures of lymph node cells and the content of IL-4 in lymph node cells were determined. LF had a profound inhibitory effect on the eliciting phase of the immune response to oxazolone as measured by the ear edema and lymph node cell number. The suppressive effect of LF on the effector phase of the immune response to TDI was moderate. LF had some stimulatory effect on the *ex vivo* content of IL-4 in lymphocytes in the immune response to TDI. On the other hand, it significantly inhibited IL-2 *in vitro* production in the immune response to oxazolone. The data strongly suggest that LF exerted differential actions on the activities of antigen-specific Th1 and Th2 cells involved in respective types of the cutaneous immune responses.

Effects of lactoferrin on allergen-induced pleurisy in mice

We also assessed the utility of LF to restrain allergen-induced pleurisy in BALB/c mice. Mice were immunized intraperitoneally with ovalbumin (OVA) and the pleurisy was elicited 14 days later by intrapleural injection of OVA. LF was given 24 and 3 h before elicitation of the allergic reaction. The cytokine levels in the pleural exudates were measured by immunoassays. The blood and pleural exudate smears were reviewed histologically. Lung sections were stained with eosin and hematoxylin for histological evaluation. LF significantly decreased manifestation of pleurisy induced by OVA in a sensitized mouse model. In particular, the percentages of eosinophils in blood and pleural exudates were strongly diminished. The histological analysis of lungs revealed that LF diminished the development of pathological lesions, such as pulmonary edema, diffuse alveolar hemorrhage and

hemosiderosis, which were found in the lungs after injection of the eliciting dose of OVA. LF also decreased the level of IL-5 secreted into the pleural fluid. This is the first demonstration that LF significantly decreases antigen-specific pleurisy in a sensitized mouse model.

A novel immunosuppressive peptide originating from the ubiquitin sequence

Ubiquitin is a conservative polypeptide present in every eukaryotic cell. Apart from its involvement in proteasomal degradation and other intracellular signal pathways, it was suggested to play an important role as an extracellular immunomodulator and antimicrobial agent. Moreover, ubiquitin-derived peptides were shown to express significant biological activities. Our previous studies in a collaboration with Wrocław University revealed that ubiquitin and its decapeptide fragment with the LEDGRTLSDY sequence, located on the exposed molecule loop, strongly suppressed the immune response. This suggested that the loop may serve as a functional epitope of the ubiquitin molecule and that a possible mechanism of biological action of the synthesized peptides is associated with interfering in interactions of ubiquitin with other molecules. Ubiquitin is known to exist in oligomeric forms, which can interact with various oligomeric receptors. New dimeric analogs of the ubiquitin fragment were synthesized, to probe whether dimeric peptides may have higher affinity towards the ubiquitin receptors responsible for immunosuppression, which are believed to form oligomeric structures. Three dimerization strategies, N-terminus to N-terminus, C-terminus to C-terminus, and N-terminus to C-terminus (head-to-tail) via PEG derivatives, were used to synthesize the dimeric peptides on a solid support. In the course of our research, we developed a new and straightforward procedure of dimerization where α-amino groups of the C-terminal lysine residues of two peptide fragments were linked by a PEG spacer directly on a solid support. The effects of dimeric analogs on the immunological response were tested in the humoral immune response to sheep erythrocytes (SRBC) *in vitro*. The results showed that the head-to-tail dimerization caused a more profound increase in the biological activity than other tested dimerization methods. Our results suggest that such orientation of peptide components may correspond to orientation of the hypothetical ubiquitin receptors responsible for the immunomodulatory activity.

Restoration of immune system function is accelerated in immunocompromised mice by the B-cell-tropic isoxazole R-11

Restoration of impaired immune response in immunocompromised patients is a crucial problem. We evaluated the efficacy of isoxazole R-11 in reconstitution of the immune

response in immunosuppressed mice. Mice were given a sublethal dose of cyclophosphamide (CP). The cellular immune response to ovalbumin (OVA) and the humoral immune response to SRBC were generated. R-11 was administered at repetitive, intraperitoneal doses (20 μg/mouse) until determination of the immune responses: 7 and 15 doses on alternate days for the cellular and humoral immune response, respectively. For phenotypic studies R-11 was given *per os*, at a single dose of 20 μg/mouse. The ability of R-11 to affect interleukin-6 (IL-6) production was determined in the whole human blood cell culture. R-11 increased the content of CD19+ cells in the spleens and lymph nodes with a concomitant decrease of CD3+ and CD4+ cells. The compound significantly accelerated restoration of both cellular and humoral immune responses, elevated the numbers of circulating leukocytes and splenocytes and normalized the blood cell picture. Supplementary experiments showed that R-11 was not toxic with regard to human peripheral blood mononuclear cells (PBMC) and that it upregulated IL-6 production in blood cell culture stimulated with lipopolysaccharide. In summary, we demonstrated that R-11 is likely a B-cell tropic agent which can restore both cellular and humoral immune responses in immunocompromised mice and may have a potential to be applied in therapy of immunocompromised patients.

Bone cells – osteoblasts, chondrocytes, and fibroblasts – as a bridge between the immune system and a source of cells for regenerative medicine

The aim was to investigate the ability of selected bone cells to maintain the correct phenotype and to secrete biologically active morphogenetic protein (BMP – bone morphogenetic protein). The study was conducted mainly on human chondrocytes derived from the Regional Tissue Bank at the Blood Donation Center in Katowice, which were not used during the treatment of autologous reimplantation, stabilized commercial chondrocyte cell lines and fibroblasts. It was shown, using various techniques, that these cells: (i) possess active BMP-2 and BMP-4 genes, (ii) synthesized and secreted biologically active BMP able to activate specific receptors presented on effector cells, which is manifested by synthesis of the specific signaling molecules phosphorylated Smad proteins and alkaline phosphatase, (iii) differ significantly in the expression of BMP-2 and BMP-4. Moreover, the presence of mRNA for BMP-specific receptor of RANKL was documented in the cells. Synthesis of biologically active BMP by human chondrocytes and fibroblasts and demonstration of mRNA expression for the receptor is the basis to believe that the stabilization of the typical characteristics of these cells during the long propagation before reimplantation surgery may depend on the mechanism of autocrine regulation of these cells. Moreover, these features of

fibroblasts allow the cell signal transmission between the cells of the bone system, as well as between the immune system and the skeletal system. All these findings have applicable aspects and address issues of regenerative medicine.

Study on the effect of nickel ions on the condition and functioning of human osteogenic cells

The studies were conducted on pigs in cooperation with the Department of Maxillofacial Orthopedics and Orthodontics, Wroclaw Medical University. Nickel ions from orthodontic appliances showed discrete, adverse effects on human osteoblasts. The oral environment is conducive to corrosion, and favorable for distribution of electrolyte ions released during this process. Twenty-four pigs were used for the experiment. Twelve of them received a metal implant consisting of the same material as used in dental braces, which was placed on the inner side of the cheek. The preliminary assessment was made on the basis of two parameters: first, describing the condition of the culture, i.e. cell proliferation and viability; and second, based on PBMC proliferation assay. It showed a significant increase in the proliferation of mononuclear cells of peripheral blood from animals treated with nickel ions in comparison with the control group.

Laboratory of Immunopathology
Head: Professor Irena Frydecka, M.D.
Studies on the mechanisms of immune deficiency in neoplastic and autoimmunological diseases

Association study of novel co-inhibitory molecule BTLA gene polymorphisms with susceptibility to multiple sclerosis

MS is a chronic inflammatory demyelinating disease of the central nervous system which has T-cell mediated etiology. The B- and T-lymphocyte attenuator (BTLA) is a novel lymphoid receptor that inhibits lymphocyte activation. The single nucleotide polymorphism (SNP) *BTLAc.800G>A* was shown to be associated with susceptibility to rheumatoid arthritis (RA) in the Taiwan population, while *BTLAc.590A>C* SNP confers risk of RA in the Japanese population. An extended study was undertaken to evaluate the association between the polymorphisms *BTLAc.800G>A* (rs9288952), *BTLAc.590A>C* (rs76844316), *BTLAc.88+384C>T* (rs1844089) and susceptibility to MS. Genotyping was done using allelic discrimination with the TaqMan®SNP Genotyping Assays in 228 MS patients and in 418 controls.

For the first time we determined polymorphisms of the *BTLA* gene in a Caucasian population. Distribution of alleles and genotypes for investigated SNPs in patients and

controls was similar: *BTLAc.88+384C>T*: allele: G– 93.1% vs. 91.4%, A– 6.9% vs. 8.6%; genotypes: GG– 86.3% vs. 83.3%, GA– 13.7% vs. 16.2%, AA– 0.0% vs. 0.5% and *BTLAc.800G>A*: allele: A– 94.7% vs. 94.2%, G– 5.3% vs. 5.8%; genotypes: AA– 89.4% vs. 88.9%, AG– 10.6% vs. 10.6%, GG– 0.0% vs. 0.5%.

The *BTLAc.590A>C* site seems not to be polymorphic in the Polish population, since all typed individuals (100 pts and 100 controls) were GG homozygous. As a result, this study did not find any genetic contribution of the BTLA gene to the development of MS in the Polish population.

Exogenous IL-2 controls the balance in Th1/Th17/Treg cell distribution in patients with progressive rheumatoid arthritis treated with TNF-alpha inhibitors

Th1/Th17/Treg imbalance dependent on cytokine profile in PB may be responsible for development and/or progression of rheumatoid arthritis (RA). The hypothesis that exogenous IL-2 is capable of controlling the balance within the CD4+ T cell pool in progressive RA was tested in 36 RA patients and 15 healthy controls. RA patients were using methotrexate (MTX group) or TNF-α inhibitors (iTNF group; with active/progressive RA). Studies were performed before and after 6 months of treatment. Th1/Th17/Tregs isolated from PB were determined by flow cytometry before and after 48 h culture with OKT3 and OKT3+rIL-2. Cytokines serum levels were analyzed by using CBA.

Before treatment, IL-6 levels and the PB Th17 subset were increased in all patients. In the iTNF group, we additionally found lower Th1 cytokine amounts, and diminished Th1 and Treg subpopulations. 48-h stimulation normalized phenotypic disturbances except for an up-regulated Th17 subset in all patients. Therapy did not normalize IL-6; the iTNF group still exhibited defective Th1 cytokines. Normalization of Th17 subsets in all patients and Tregs in the iTNF group was accompanied by defective Treg function and a lower Th1 subset in the iTNF group. 48-h re-stimulation decreased the Treg population in the iTNF group, only reversed by exogenous IL-2 addition to the culture.

Patients with progressive RA present many immune alterations in PB compared to the MTX group, of which systemic Th1 defects seem to be irreversible, supporting the role of disease progression in subsequent changes. In these patients, exogenous IL-2 is capable of promoting Treg differentiation disturbed by chronic stimulation, suggesting its use in combined strategy of biological treatment.

Laboratory of Reproductive Immunology
Head: Professor Anna Chełmońska-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.

Uterus gene transcription regulation

During the pre-implantation period of pregnancy mutual interaction between the conceptus and pregnant female are possible due to secretory activity and direct interactions between the oviduct and uterus epithelial cells and embryo cells. The strength and quality of embryonic signals are decisive for pregnancy maintenance. The presence of the embryo evokes transcriptional changes in the pre-implantation uterus and genes of signal transduction pathways are also regulated during this period of pregnancy. The aim of the study was to analyze the expression of genes of main pathways of signal transduction in uteri of females in 3.5 days of pregnancy in comparison to pseudopregnant mice. Females of CD1 were synchronized by administration of FSH and LH containing medicines and mated by fertile males. Pseudopregnancy was achieved by mating females with vasectomized males. Expression of 79 genes from 18 signal transduction pathways was analyzed by RT² Profiler PCR Array Mouse Signal Transduction Pathway Finder (SA Bioscience). It was shown that in the uterus of pregnant mice expression of most analyzed genes was down-regulated and among them the expression of 22 genes was silenced over 5 times. Expression of only 7 genes increased up to two-fold compared to the control group. Among them the COX-2 gene transcription level was up-regulated two-fold. The results suggest that embryonic signals silence many genes involved in different signal transduction pathways but COX-2 – a gene critical for pregnancy maintenance – stayed positively controlled.

Protein expression in lymphocytes

The involvement of the peripheral compartment of the immune system during early pregnancy is poorly understood. It was established during former studies performed in the Laboratory of Reproductive Immunology that spleen antigen presenting cells in mice during the pre-implantation period of pregnancy enhance expression of costimulatory molecules and therefore may influence lymphocyte activity. Proteomic analysis of spleen CD4 lymphocytes was performed in pregnant and pseudopregnant mice. Females of CD1 mice were mated with fertile males or with vasectomized males. At 3.5 days after mating the mice were euthanized and spleens were recovered. CD4 lymphocytes were isolated after magnetic sorting (CD4 + T

Cell Isolation Kit, MiltenyiBiotec MACS). Isolated CD4. Purity of isolated cells was equal to ca 80%. Cells were lysed under protection of protease inhibitors, centrifuged and the supernatant was frozen at -80°C. Subsequently, proteins were separated using 2D electrophoresis. Gel protein spots were stained and cut from the gel and analyzed in a MALDI-TOF mass spectrometer. 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. The difference in expression of 25 proteins in samples from spleens of pregnant and pseudopregnant mice was observed. Five identified proteins are involved in regulation of cell motility.

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

Laboratory of the Molecular Biology of Microorganisms
Head: Professor Jolanta Zakrzewska-Czerwińska, Ph.D.
Helicobacter pylori oriC – the first bipartite origin of chromosome replication in Gram-negative bacteria
Home page: www.iitd.pan.wroc.pl/dept/mic/index.htm

DNA synthesis is tightly controlled and strictly dependent on cell cycle progression. It is primarily regulated at the first step, which is initiation. Most of the information about bacterial chromosome replication comes from studies on *Escherichia coli*, whose crucial initiation elements – origin of chromosome replication (*oriC*) and the initiator protein (DnaA), as well as accessory and regulatory factors – have been thoroughly characterized. The initiation of chromosome replication is much less understood in bacteria other than *E. coli*. Though the basic mechanism of the process is believed to be conserved in all microorganisms, the recent studies indicate unique features of the initiation factors in various bacteria, which make the initiation of chromosome replication similar in related bacteria while remaining distinct between unrelated species. Examples of such unique features are the bipartite structure of the *oriC* region and the topology-sensitive DnaA-*oriC* interactions recently discovered in *H. pylori*. The *H. pylori oriC* is composed of two subregions – *oriC*1 and *oriC*2 – separated by the *dnaA* gene. It resembles the split origins described only for a few mollicutes and Gram-positive firmicutes, but never for a Gram-negative bacterium. Moreover, the *H. pylori* mode of DnaA-*oriC* interaction, with the loop formation between the *oriC* subcomplexes, resembles that characterized in *B. subtilis* and in many plasmids, which might suggest a similar, protein-deficient way of either building the initiation complex and/or controlling the initiation of replication. Surprisingly, *oriC*2 is bound exclusively as a

supercoiled DNA, which directly shows the importance of the DNA topology in DnaA-*oriC* interactions, similarly as previously presented only for initiator-origin interactions in Archaea and some Eukaryota. We assume that our results not only indicate a specific mode of regulation of *H. pylori* chromosome regulation, but may also suggest the more general way of controlling *H. pylori* cellular processes. *H. pylori* – a slowly growing bacterium, deficient in regulatory proteins, living in an invariant ecological niche – may use more basic regulatory levels such as epigenetics (e.g. DNA topology and looping) instead of multiple protein regulators to regulate its cellular functions.

Laboratory of Signaling Proteins
Acting Head: Professor Janusz Matuszyk, Ph.D.
Studies on proteins and signaling pathways involved in the activation of proinflammatory transcription factors and response to hypoxia

Adenosine is produced intracellularly and in the intercellular space of adenine nucleotides. Under conditions of oxidative stress and damage to the cells, the concentration of adenosine is increased from nanomolar to millimolar values. Adenosine induces response to hypoxia, including biosynthesis and release of catecholamines from the cells of the adrenal medulla. A key step in the biosynthetic pathway of catecholamines is catalyzed by tyrosine hydroxylase (TH) and hypoxia induces expression of the tyrosine hydroxylase gene. A sudden increase in blood pressure induced by catecholamines leads to increased secretion of atrial natriuretic peptide (ANP) from the heart into the circulatory system. ANP could act as a negative regulator of adenosine-induced transcription of the tyrosine hydroxylase gene.

The PC12 cell line has been derived from rat pheochromocytoma and that cell line is used to study signal transduction in neural and adrenal medulla cells. The cellular effects of adenosine are mediated through specific G-protein coupled receptors located on the cell surface. PC12 cells express the A2A adenosine receptor, which stimulates adenylate cyclase activity. Increased production of cyclic AMP (cAMP) then leads to activation of the TH gene promoter in reporter assays and increased TH mRNA level in PC12 cells. PC12 cells also express on the cell surface natriuretic peptide receptor, which is a membrane-bound guanylyl cyclase type A (GC-A). ANP binds to the extracellular domain of GC-A, thereby activating guanylyl cyclase and increasing cyclic GMP (cGMP) production.

The results of our experiments show that treatment of PC12 cells with ANP reduces activation of the TH gene promoter and transcription of the TH gene in response to stimulation with adenosine. Measurements of intracellular cAMP and cGMP levels in PC12

cells show increase in cGMP levels in response to stimulation of ANP as well as an increase in cAMP levels in response to stimulation with adenosine alone and a lower increase in cAMP levels in cells treated with adenosine and ANP. This suggests a reduction in cAMP level in response to an increase in cGMP level, presumably as a result of hydrolysis of cAMP by phosphodiesterase 2 (PDE2), which is activated by cGMP. The results of experiments with the use of EHNA (a pharmacological inhibitor of PDE2) indicate the involvement of PDE2 in signal transduction from receptor of ANP to inhibition of adenosine-induced activation of the TH gene in PC12 cells.

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

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

In the year 2012 we concluded multicenter research activity in the field of immunogenetics of donor-recipient matching for HSCT. In this activity 11 institutions (7 clinical departments and 4 laboratories) took part, contributing with 360 unrelated donor-recipient pairs. The aim of this project was twofold: (i) standardization of HLA and (ii) evaluation of the usefulness of cytokine/chemokine gene polymorphism as risk factors of the outcome of HSCT.

Within this project the following novel information was documented:

1. Matching at different loci has a different weight in affecting the survival of patients. Locus A and DR specificity mismatches are the most deteriorating, with a less significant impact of locus B then C mismatches. Locus DQ mismatch as the only one making the transplant discordant is of negligible value.
2. Prolonged process of donor search negatively affects survival of HSCT patients.
3. Functional polymorphism of TNFalpha and IL-6 genes influences the risk of toxicity and that of IL-10 and IFN-gamma has an impact on the alloreactive process. In brief: TNFalpha A2 (-308) allele and IL-6 G/G (-174) homozygosity constitute risk factors of severe toxicity after myeloablative HSCT conditioning. IL-10 gene ACC haplotype associates with lower incidence of aGvHD and this beneficial effect is highlighted by the observation of the presence of a larger pool of lymphocytes expressing FoxP3 after *in vitro* stimulation in ACC haplotype carriers. IFNgamma allele homozygosity of donors known to be associated with rather poor cytokine generation makes recipients more susceptible to aGvHD and, notably,

recipients having the allele of low IFNgamma producer suffered more frequently from CMV reactivation.

4. Comparative genomic hybridization was employed in a genetic analysis of 53 AML patients. It was shown that karyotype normal patients had obscured several genetic abnormalities, namely depletion and/or amplifications in chromosome regions having genes known to be associated with malignancies. It will help in more precise genetic description of the cases, which makes it possible to use chemotherapy tailored to the case.

The above examples of the results of the project coordinated by our institute provide a rationale beyond the understanding of the genetic background to the alloreactivity and the results may be directly translated to the clinic to benefit HSCT transplanted patients.

In an effort to complete our study conducted in the frame of the institute own supporting activity we came to the following conclusions:

1. Herpes and Polyoma virus reactivations are seen in HSCT patients in a sequence as time elapses with HHV-6 appearing first followed in a time-dependent manner by EBV and CMV, with the last events associated with Polyoma BK and JC viruses. HHV-6 had the most deteriorating effect, affecting the lymphocyte recovery in the long term. CMV survivors were characterized by a good CD8+ lymphocyte response and EBV constitutes a risk factor of pure red cell aplasia and lymphoproliferative syndrome. Polyoma viruses reactivation is of a poor prognosis as seen in patients with a high-grade post-transplant immunodeficiency heralding aspergillosis. These data if used for developing a strategy of post-transplant surveillance may be useful in planning proper prophylactic and pre-emptive measures.

2. Final analysis of Treg cell presence in the post-transplant lymphocyte pool. We came to the important conclusion that the level of Tregs at the time of hematological recovery shapes the final outcome of transplantation in a long-term perspective. This conclusion was drawn from the observation of survivors and also fatalities after transplant. High Treg cell patients survive better but if the course is fatal it is more often associated with a deteriorating chronic GvHD.

3. Fortunately by the end of 2012 we completed construction of the GMP facility which opens new possibilities to begin practical application of cell therapy, giving hope for better dealing with cancer and infections.

We were proud organizers of the conference on „PRACTICAL ASPECTS OF DONOR-RECIPIENT MATCHING IN HSCT" with great participation of a number of leading scientists in the field of immunogenetics. Also in line with this activity was our editorial work in a volume entitled *Biological and Genetic Aspects of Donor-Recipient Matching in HSCT*, recently published by Hindawi Publishing Corporation.

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

Killer immunoglobulin-like receptors (KIRs) are expressed on NK cells and also on some T lymphocytes. They may inhibit or activate a cell upon binding a ligand. The majority of known KIR ligands are HLA class I molecules, and normal cells of the body are not killed by NK cells because their (self) HLA molecules are recognized by inhibitory KIRs. However, neoplastic cells may lose HLA expression, and therefore they may be eliminated by NK cells. The KIR repertoire of NK cells of an individual depends on presence or absence of *KIR* genes, which are distributed differently in individuals in a population (haplotypic polymorphism). The repertoire of inhibitory and activating KIRs influences the capacity of the immune system to eradicate cancer cells. We examined the effect of *KIR* genotype in non-small cell lung cancer (NSCLC). No *KIR* gene was associated with susceptibility to NSCLC. However, patients positive for *KIR2DL2* and *KIR2DS2* genes and homozygous for their ligand, a C1 epitope of HLA-C molecules, were 6 times more likely to respond to treatment than those with other genotypes, and survived longer than others.

In transplantation of allogeneic organs, even if ideally HLA-matched, the KIR repertoire may differ between donor and recipient, because *KIR* and *HLA* genes are located on different chromosomes. We performed a study on *KIR* gene associations with kidney graft rejection. We found that in recipients whose end-stage renal failure had a cause other than glomerulonephritis, *HLA-B,-DR* matching had a stronger effect on graft rejection than *KIR* genes. However, in patients whose end-stage renal failure was caused by glomerulonephritis, *HLA* compatibility seemed to be much less important for graft rejection than the presence of the *KIR2DS4* gene. In addition, the *KIR2DS5* gene seemed to protect the graft from acute rejection in the presence of the full-length *KIR2DS4* gene but in the absence of its variant with 22 base pair deletion.

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

Laboratory of Molecular and Cellular Immunology
Acting Head: Professor Małgorzata Cebrat, Ph.D.
Lymphoid-specific inactivation of NWC promoter is regulated through antisense-mediated transcription and Ikaros transcription factor

Expression of the *NWC* gene, which is the third evolutionarily conserved gene in *RAG* (*RAG-1* and *RAG-2* locus), is regulated by a promoter localized in the *RAG-2* intron and becomes inactivated in immature and mature lymphocytes. We have shown that inactivation of the *NWC* promoter is caused by the methylation of a CpG island which is associated with the 5'- end of the *NWC* gene. We hypothesized that the lymphoid-specific methylation of the *NWC* promoter can be caused by the expression of *RAG-2* transcript (which is both antisense towards *NWC* expression and lymphoid-specific) and/or binding of the promoter to a lymphoid-specific transcription factor which could act as a transcriptional repressor. In order to verify our hypothesis, we obtained a transgenic mouse model – the inserted transgene was a full-length *RAG/NWC* locus obtained from a bacterial artificial chromosome modified to create *RAG-2/GFP* and *NWC/YFP* fusion genes and with a transcriptional termination cassette inserted immediately downstream of the first exon of the *RAG-2* gene. We obtained 4 transgenic precursors. The offspring were analyzed to choose mice with the most efficient downregulation of *RAG-2* expression in developing B- and T lymphocytes. The methylation level of the transgenic *NWC* promoter methylation level was then assessed using bisulfite sequencing. 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 have also shown that the Ikaros transcription factor, which can act as a transcriptional repressor, may also downregulate activity of the *NWC* promoter. The *NWC* promoter has two Ikaros-binding sites which are shared with ZFP-143 transcriptional activator. We have shown, by EMSA and reporter assays, that overexpression of Ikaros leads to reduced ZFP-143 binding and downregulation of activity of the *NWC* promoter, probably due to outcompeting of ZFP-143 for binding to the promoter.

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 Laboratory of Tumor Immunology focuses on investigating the role of the LAT (linker for activation of T cells) adaptor in transducing cell survival or cell death promoting signals to various subsets of normal or leukemic T cells. In this context we collaborated with Dr Enrique Aguado's laboratory (Cadiz, Spain) in describing for the first time a process of caspase-dependent proteolytic degradation of LAT in response to T-cell receptor (TCR) or

Fas receptor signaling. In the framework of this project we have mapped amino acid motifs of LAT that are cleaved by caspases and we have also evaluated signaling capacity of an LAT mutant resistant to proteolytic degradation. Since the cleavage sites of LAT are located in close proximity to a number of important C-terminal regulatory tyrosine-based motifs, we assessed how tyrosine phosphorylation affects proteolytic cleavage. It turned out that persistent phosphorylation of LAT prevented its degradation even upon strong pro-apoptotic treatment of thymocytes and a thymoma cell line. This finding suggested a possible mechanism for regulating the strength and duration of LAT mediated signals.

In the framework of the above-mentioned project we also developed a flow cytometry based assay for single cell measurement of LAT proteolytic degradation. Using this assay we found that FoxP3+ thymocytes (i.e. precursors of thymus derived regulatory T cells) are resistant to LAT degradation in response to strong TCR signals normally leading to negative selection of a majority of FoxP3- thymocytes. This finding may be used as an additional, functional marker of T regulatory cell lineage.

Our method of assessing LAT degradation may also be used to measure the status of T cell mediated immune response in health and disease. In particular it may be useful in following the progression of T cell mediated autoimmune conditions or anti-tumor response.

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

Research done: In collaboration with Dr Claudine Kieda from Centre Biophysique Moleculaire (CNRS, Orleans, France) we have established two cell lines of human early endothelial progenitor cells with retained high proliferative potential (HEPC-CB.1 and HEPC-CB.2). Experiments were designed to determine the differentiation conditions of both

cell lines into mature vascular endothelium. Cells cultured on fibronectin coated plates, with optimal concentrations of dibutyryl-cAMP, retinoic acid and VEGF, reduced the expression of progenitor cell markers (CD271, CD 133, CD90). We also observed increased expression of the CD146 marker, characteristic for endothelial cells. There was no expression of mature endothelial cell markers (CD31 and von Willebrand factor). Hypoxia had no effect on the rate of the differentiation process of both cell lines.
Research carried out in collaboration with Wrocław Medical University clinics addressed the characteristics of circulating endothelial progenitor cells in pregnancy-induced hypertension. The results were submitted for publication.

The use of the results: The study may contribute to the knowledge of the differentiation process of progenitor cells to mature endothelium, which may help to determine their regenerative potential and allow the subsequent use of these cells in the treatment of diseases in the case of endothelial injury, such as myocardial infarction.

The role of UDP-galactose:ceramide galactosyltransferase (UGT8) and galactosylceramide (GalCer) in breast cancer progression

The enzyme UDP-galactose:ceramide galactosyltransferase (UGT8) is responsible for the synthesis of galactosylceramide (GalCer). Using transcriptome profiling, it was shown that *UGT8* is one of six genes whose elevated expression correlated with a significantly increased risk of lung metastases in breast cancer patients. Our recent studies using immunohistochemistry and real-time PCR on the presence of UGT8 in breast cancer tissue specimens revealed a significant increase in UGT8 expression in (1) lung metastases in comparison to primary tumors, (2) tumors of malignancy grade G3 in comparison to tumors of malignancy grades G2 and G1, (3) node-positive tumors in comparison to node-negative tumors. The predictive ability of increased expression of UGT8 was validated at the mRNA level in three independent cohorts of breast cancer patients. Therefore, our data suggest that UGT8 can be a significant index of tumor aggressiveness and a potential marker for the prognostic evaluation of lung metastases in breast cancer. In addition, we found that the "mesenchymal-like" cells MDA-MB-231, BO2, and MCF10CA1a.c11, each forming metastases in nude mice, are the only cell lines synthesizing GalCer.

Very little is known about the possible functions of GalCer in tumor cells, including breast cancer cells. Therefore, the role of UGT8 and GalCer in tumor growth and formation of experimental metastases was studied *in vivo* in athymic nu/nu mice. It was done by

constructing a specific “loss-of-function” phenotype represented by MDA-MB-231 cells transduced with small hairpin (sh) RNA targeted UGT8 mRNA (MDA/LUC-shUGT8 cells). It was found that control MDA-MB-231 cells transduced with vector alone form tumors much more efficiently in comparison to MDA/LUC-shUGT8 cells with suppressed synthesis of GalCer after orthotopic transplantation. In accordance with this finding, immunohistochemical staining of tumor specimens revealed that high expression of UGT8 accompanied by accumulation of GalCer in control MDA-MB-231 cells is associated with a much higher proliferative index and lower number of apoptotic cells in comparison to the MDA/LUC-shUGT8 cell line. It was also found that breast cancer cells expressing higher levels of UGT8 and synthesizing larger amounts of GalCer revealed higher ability to form metastatic colonies after intracardiac inoculation into nu/nu mice. Taking the results together, the suppression of UGT8 expression in MDA-MB-231 cells has a profound effect on their tumorigenic and metastatic properties.

Publications – 2012

Articles published in the journals from Thomson Reuters Master Journal List:

1. Artym J.: A remedy against obesity? The role of lactoferrin in the metabolism of glucose and lipid. (Złoty środek na otyłość? Udział laktoferryny w metabolizmie glukozy i lipidów). *Post Hig Med Dośw, (online)*, 2012; 66, 937-953 IF – 0,654 (15 pkt Min.)
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