Ludwik Hirszfeld Institute of Immunology and Experimental Therapy
Polish Academy of Sciences
Rudolfa Weigla 12, 53-114 Wrocław

Research Report 2010

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

Research on the biology of bacteriophages and their use in the treatment of bacterial infections

An investigation of the bacterial profile of microbiological samples taken from patients with chronic infections, treated at the Phage Therapy Unit in Wrocław, was carried out. The results of the identification and quantitative cultures of the bacterial strains isolated from 13 patients with wound infections (caused by *S. aureus* in 10 cases, *P. mirabilis* in 1 case, *P. aeruginosa* in 1 case, and mixed *S. aureus/P. aeruginosa* infection in 1 case) before, during and after completion of the phage therapy were analyzed. A decrease (by 1-4 log) in the number of bacteria in analyzed samples, accompanied by a clinical improvement, was observed in 46% of patients.

The study on the influence of immunosuppressive and anti-inflammatory drugs on the spontaneous and induced lysogeny of *E. coli*, *S. aureus*, and *E. faecalis* strains showed no effect of sirolimus (20-100 ng/ml) or hydrocortisone hemisuccinate (0.1-1.0 μg/ml) on the induction of phages from the tested bacteria.

The prevalence of coliphages in stools of healthy volunteers and patients with inflammatory bowel diseases such as Crohn's disease and colitis ulcerosa was investigated. Stools from 10 healthy volunteers, 10 patients with Crohn's disease, and 36 patients with colitis ulcerosa were analyzed. Strains of *E. coli B*, *E. coli 1962*, and in some cases *E. coli DSM 13127* and the strain of *E. coli* isolated from the patient were used for phage detection. It was observed that coliphage frequencies were lower in the stools of patients as compared to healthy individuals. The mean titer of coliphages was greater in patients than in healthy volunteers. The mean *E. coli* concentration was also greater in patients (6.9×10^7 CFU/g) than in healthy volunteers (1.1×10^7 CFU/g). However, these differences did not reach statistical significance.

A preliminary study on phage penetration into the urine in 14 phage-treated men with genitourinary tract infections revealed that applied rectally specific phages could be detected in 21.4% of cases.

An investigation of bacteriophage neutralization properties of human serum was initiated. It was observed that 35.7% of sera (only undiluted or diluted 1:10 samples) from healthy volunteers (n=14) could neutralize T4 phage. In patients (n=6) undergoing phage therapy due to *S. aureus*, *E. coli*, and *P. aeruginosa* infections the anti-phage activity (against specific phages used for

treatment) was observed only in undiluted or 1:10 diluted sera in all cases before the beginning of therapy. During the treatment this activity significantly increased in 2 patients – it was detected for serum dilution of 1:100 or even 1:1000.

Directed mutagenesis of T4 phage was performed. In the first step a recombination vector with *amber* codon in the area of the KGD motive in the gene *24* was prepared (mutagenizing primer method). The first recombination step was driven by suppressive cytosolic conditions of *E. coli* SupF strain. Selection for plaque-forming ability was done in the non-suppressive strain. Next, vectors with a designed mutation were constructed (mutagenizing primer method) and the second recombination and selection resulted in phages T4 and HAP1 that have the KGD region in gp24 changed to WGD, KYD, and/or RGD.

Parallel investigation of isolated bacteriophage proteins comprised GFP-marker construction for *in vivo* (athymic mice) and *in vitro* (confocal microscopy) investigations of the protein circulation. Sequential cloning (combined restriction and two-step recombination) was used for preparing GFP fusions of T4 head proteins GFP-Hoc, GFP-Soc, GFP-23, and GFP-24 with the affinity purification tags (removable by proteolysis). All the products were effectively expressed, isolated and purified, and an intensive GFP signal was confirmed. Preparations were applied in preliminary *in vivo* circulation tests, which showed tissue accumulation of the tested fusion proteins.

Results of grant activities

Expression of bacteriophage capsid proteins from expression vectors was conducted. The genes used for expression were: hoc, soc, 24 (the cleaved form), 23 (the cleaved form), 11, 12, 18, 36, 37, and wac. New plasmid constructions for gp12 and gpSoc were prepared. Effective expression including chaperons (coexpression) allowed us to obtain recombinant proteins with amino acidic/protein motifs able to bind specific slurries and to perform chromatographic purification of the proteins (native, non-denaturizing conditions). Proteolytic release as an "in-slurry" reaction, LPS-affinity chromatography (EndoTrap), and FPLC processes were used for purification. Approximately 1-2 mg/ml of the high-purified protein preparations were used for *in vitro* and *in vivo* activity assays: cell migration and adhesion, metastatic potential, massive cytokine arrays, and ROS production. The preliminary experiments indicated adhesive properties of two T4 head proteins: gp23 and gp24.

Testing of 157 new environmental samples resulted in isolation of 14 *Klebsiella pneumoniae* phages, 9 *Klebsiella oxytoca* phages, 11 *Morganella morganii* phages, 8 *Enterobacter cloacae* phages, 2 *Enterobacter aerogenes* phages, 3 *Enterobacter gergoviae* phages, 6 *Enterococcus faecalis* phages, 2 *Pseudomonas aeruginosa* phages, 4 *Citrobacter freundii* phages, 1 *Escherichia*

coli phage, and 1 *Acinetobacter baumannii* phage. The most effective source of the phages proved were incubated and condensed samples of crude sewage as well as hospital sewage. Studies on the biological activity of recently isolated phages showed the unfavorable influence of temperature and chloroform on the lytic activity of *E. coli*, *E. faecalis*, and *S. maltophilia* phages. Morphological and ultrastructural analysis of new *Stenotrophomonas* and *Citrobacter* phages lead to their classification to *Myoviridae*, *Siphoviridae* and *Podoviridae* families of *Caudovirales*.

Our investigations showed no substantial influence of *S. aureus*, *E. coli*, and *P. aeruginosa* bacteriophage preparations on the migration properties of human immune cells *in vitro*. Only in a very few cases of *S. aureus* preparations, inhibition or stimulation of migration was observed.

A possibility of phage penetration into mice blood and rat prostate after rectal phage application was confirmed. Moreover, the addition of non-ionic surfactants to the phage lysate significantly increased the phage titer in blood. Although we could not confirm the phage ability to penetrate from the intestine into the rat blood, we observed successful phage penetration into the blood of mice orally treated with phages, but it required application of stomach acid neutralizers or inhibitors.

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 main research topics in our laboratory are mechanisms of pathogenicity of diseases with bacterial etiology, the role of bacterial proteins and glycoconjugates in pathogenicity, and structure and functions of bacterial exopolysaccharides and endotoxins. In the framework of the studies of molecular markers of infectious diseases and biochemical factors specific for inflammatory processes a method has been elaborated for quantitative determination in serum samples of four different markers specific for Gram-negative and Gram-positive bacteria. The determination of such chemical markers in combination with immunoenzymatic assays will improve diagnostic analyses in clinical practice, allowing monitoring of the clinical pattern of sepsis and septic shock. Regarding the difficult problem of identification of actinomycetes clinical isolates, their glycolipids and mycolic acids as well as exopolysaccharides appeared to be useful markers which facilitate diagnostic procedures. Probiotics are considered to have a potential in protection against invading bacteria, but the mechanisms of this process are not well understood. In order to study the role of probiotics in inflammatory bowel disease, where probiotic

microorganisms are also involved, we determined the structure of exopolysaccharides produced by two strains of *Lactobacillus reuteri*. The strain isolated from mice with experimentally induced inflammatory bowel disease produced teichoic acid like structure, whereas exopolysaccharide isolated from *L. reuteri* strain from healthy mouse contained only polysaccharide found previously in *L. rhamnosus* milk strain and also in a strain of human origin. The project of studies on advanced glycation end-products (AGE) concerned the elaboration of a method for preparation of AGE using a microwave reactor and purification protocol. These glycation products have proved to be useful antigens for determination of antibodies present in serum of diabetic patients.

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

The replication of vesicular stomatitis virus (VSV) in isolated human leukocytes has been used to measure the level of nonspecific antiviral immunity. However, during infection with some pathogens, the main effect observed is caused by interaction between the pathogen and VSV. This was also noted in advanced stages of HIV infection, when an inverse association between HIV viral load and VSV replication was found. The mutual effect was markedly stronger than the correlation between the VSV replication level and CD4ț T-cell count. Since successful antiretroviral therapy is associated with a decrease in HIV viremia to undetectable levels, the effect of such therapy on VSV replication was expected and confirmed in this investigation. In fact, increased VSV titers were observed together with decreased HIV viral load, particularly in the case of efficient therapeutic schemes, for example those including lopinavir/ritonavir. The results showed that VSV replication capacity reflected the progression of HIV infection. Moreover, the presence of interferon in the plasma of AIDS patients was found to be only partially responsible for the inhibition of VSV replication. The results suggest a specific HIV-VSV interaction, whether direct or indirect. Thus the VSV replication assay may be applied in evaluating the stage of HIV infection. The results were published in *Viral Immunology 2010; 23: 567-576.*

Cancer disease is accompanied by inflammation, oxidative stress, strong proliferation of cancer cells, resulting with growth of tumor and its consecutive vascularization, metastases accompanied by reduction of innate immunity. The anticancer effect of *Scutellaria baicalensis* flavones was checked and discussed in view of present literature data. Inhibition of growth of different tumor cell lines by flavones isolated from root of the plant – wogonin, baicalein and baicalin, but not wogonoside – was found. The anticancer activity of the flavones was connected with reduction of high level reactive oxygen species (ROS), reduction of the inflammatory reaction, and NF-κB activation. Inhibition of the cell cycle and involvement of cyclins in the

process are discussed. The most important anticancer reaction of *Scutellaria* flavones was stimulation of apoptosis of cancer cells. The molecular mechanism of anti-prostate and anti-breast cancer cells activity of *Scutellaria baicalensis* extract and the herbal mixture PC-SPES is discussed. Flavonoids from *Scutellaria* extracts strengthened leukocyte antiviral innate immunity and modulatted cytokines production in acute leukemia leukocytes, which seems to be very important in human cancer therapy. The results were published in *Adv. Clin. Exp. Med. 2010; 19: 419-428.*

Ginkgo biloba special extract EGb 761 and donepezil are drugs used in Alzheimer disease therapy. The influence of donepezil and EGb 761 on two mechanisms of innate immunity: natural antiviral resistance of human leukocytes *ex vivo* and NF-κB activation, were studied. The correlation between the innate immunity of leukocytes and NF-κB activation was investigated. The effect of the two drugs on human leukocytes resistance to vesicular stomatitis virus (VSV) infection was also assessed. Two groups of healthy blood donors (n=30) were distinguished: one with resistant leukocytes (n=15) and one with leukocytes sensitive to VSV (n=15). The level of natural resistance of human peripheral blood leukocytes (PBLs) was determined by studying the kinetics of VSV replication. NF-κB activation was assayed by immunocytochemical staining. Efficiency of donepezil and EGb 761 was determined by a special regression model. The toxicity of the preparations to PBLs and the cell lines L929 and A549 and their effect on the different viruses was established. The results showed that donepezil used in concentrations of 10–50 μg/ml and EGb761 of 25–100 μg/ml stimulated resistance of human leukocytes. At the same concentrations both preparations decreased activation of transcriptional factor NF-κB. A correlation between innate immunity of PBLs and NF-κB activation was observed. Comparison of the effects of these two drugs showed that EGb 761 is more effective in stimulating leukocyte resistance. Donepezil and EGb 761 regulated innate immunity of human leukocytes by stimulating resistance and modulating NF-κB activation. The natural drug was more efficient in stimulating innate antiviral immunity of human leukocytes. The results were published in *Int. Immunopharmacol 2010; 10: 1505-1513.*

Various *N*-substituted benzisoselenazol-3(2*H*)-ones and their non-selenium-containing analogues have been synthesized and tested against selected viruses (HHV-1, EMCV and VSV) to determine the extent to which selenium plays a role in antiviral activity. The data presented here show that the presence of selenium is crucial for the antiviral properties of benzisoselenazol-3(2*H*)-ones since their isostructural analogues having different groups but lacking selenium either did not show any antiviral activity or their activity was substantially lower. The open-chain analogues of benzisoselenazol-3(2*H*)-one diselenides also exhibited high antiviral activity while

selenides and disulfides were completely inactive towards model viruses. The results were published in *Molecules 2010; 15: 8214-8228.*

Synthesis of glycosyl derivatives of hydroxyanthraquinones potentially useful for kidney stone patient therapy is presented. These compounds were analyzed as inhibitors of calcium oxalate crystal formation as well as substances with the ability to dissolve crystalline calcium oxalate. In addition, the effect of these compounds on real kidney stones was analyzed by *ex vivo* tests. The tests on L929 and A545 cell lines have shown that tested compounds were not cytotoxic. The results were published in *European Journal of Medicinal Chemistry 2010; 45: 1001-1007.*

Mycophenolate mofetil (MMF) is an immunosuppressive agent used in the prophylaxis of graft rejection in transplantology. Its antiproliferative effects on lymphocytes, monocytes, vascular smooth muscle cells and fibroblasts are well known, but to our knowledge there are no reports on its action on the retinal epithelial (RPE) cells in vitro. In all experiments we used mycophenolic acid (MPA), which is the biologically active form of MME. Its activity was assessed on cultures of an immortalized non-transformed cell line from a human donor (ARPE19). Cells were seeded and incubated *in vitro* with different concentrations of MPA: 0.0025 pg/ml, 0.025 pg/ml, 0.25 pg/ml, 2.5 pg/ml, 25 pg/ml and 250 μg/ml. After 24 and 72 hours of incubation, proliferative activity was assessed by 5'-bromo-2'-deoxyuridine (BrdU) incorporation into cellular DNA and the amount of cell proliferation was determined using the 3-(4.5-dimethylthiazol-2yl)-2.5-diphenyltetrazolium bromide (MTT) assay. Additionally, to determine MPA cytotoxicity, ARPE19 cells were grown to confluence and subsequently cultured in a serum-deficient medium and then, after 24 hours of incubation with different concentrations of MPA, the MTT test was performed. The BrdU assay showed a decrease of DNA synthesis activity for increasing concentrations of MPA from 0.025 μg/ml to 250 μg/ml. The number of RPE cells assessed with the MTT test decreased after exposure to drug concentrations of 25 μg/ml and 250 μg/ml after 24 and 72 hours of incubation, and additionally for concentrations of 0.25 μg/ml and 2.5 μg/ml after 72 hours of incubation. MMF influences the proliferation of immortalized ARPE19 cells without an evident cytotoxic effect. The results were published in *Klinika Oczna 2010;112: 201-204.*

The Jak/Stat pathway (Janus kinase/signal transducer, end activator of transcription) is used by many cytokines, hormones and growth factors involved in cellular mechanisms of gene expression, cellular activation, proliferation, differentiation and apoptosis. Data suggest that Jak/Stat activity is significant in the pathogenesis of rheumatoid arthritis (RA). The objective was to evaluate the expression of Jak3 and the activity of Stat3 in synovial fluid cells (SFCs) and blood leucocytes (BLs) in patients with RA and spondyloarthropathy (Spa) and compare it with parameters of disease activity used in clinical practice. Moreover, the relations between Jak3

expression and Stat3 activity were evaluated. Nineteen patients with RA and 22 patients with Spa (ankylosing spondylitis, psoriatic arthritis and undifferentiated spondyloarthropathy) were involved in the study. Healthy individuals (n = 23) were recruited as a control group. The expression of Jak3 and the activity of Stat3 were measured using the immunocytochemical method in BLs. In 11 RA patients and in 12 Spa patients expression of Jak3 and activity of Stat3 were measured in SFCs. In patients, laboratory parameters describing the disease activity were measured. X-ray images of the joints were also taken. In the RA and Spa group, the expressions of Jak3 and of Stat3 were higher than in healthy subjects. The results were published in *Reumatologia 2010; 48: 237-246.*

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

Laboratory of Transgenesis and Lymphocyte Biology
Head: Professor Paweł Kisielow, Ph.D.

Complexity of transcriptional regulation within Rag locus: identification of a second Nwc promoter region within Rag-2 intron

Nwc represents a mysterious third evolutionarily conserved gene within the *Rag* locus. Last year we analyzed the phenotype of Nwc^{tmpro1} mice, in which the *Rag2* intragenic region containing the previously identified promoter responsible for initiating transcription of *Nwc* in all cells except lymphocytes was deleted by homologous recombination. Despite strong nonlymphocyte-specific inhibition of *Nwc* transcription which runs through the regulatory region of *Rag* genes, their expression remained suppressed and no developmental, morphological, anatomical, functional, physiological or cellular defects in Nwc^{tmpro1} mice could be observed. However, careful analysis of the *Rag2* intergenic region uncovered a second evolutionarily conserved *Nwc* promoter region from which a previously unknown *Nwc* transcript can be generated in nonlymphocytes of Nwc^{tmpro1} and normal mice. These results revealed unexpected additional complexity of transcriptional regulation within the *Rag/Nwc* locus and showed that strong inhibition of *Nwc* transcription in non-lymphoid cells is well tolerated. Complete inactivation of *Nwc* is necessary to gain insight into its function at transcriptional and post-transcriptional levels.

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

Laboratory of the Molecular Biology of Microorganisms
Head: Professor Jolanta Zakrzewska-Czerwinska, Ph.D.
The molecular basis of replication and segregation of bacterial chromosomes
Home page: www.iitd.pan.wroc.pl/dept/mic/index.htm

The actinobacterial signature protein ParJ regulates ParA polymerization and affects chromosome segregation and cell division during Streptomyces sporulation*

Bacterial chromosome segregation usually involves cytoskeletal ParA proteins, ATPases which can form dynamic filaments. In aerial hyphae of the mycelial bacterium *Streptomyces coelicolor*, ParA filaments extend over tens of microns and are responsible for segregation of dozens of chromosomes. We have identified a novel interaction partner of *S. coelicolor* ParA, ParJ. ParJ negatively regulates ParA polymerization *in vitro* and is important for efficient chromosome segregation in sporulating aerial hyphae. ParJ-EGFP formed foci along aerial hyphae even in the absence of ParA. ParJ, which is encoded by *sco1662*, turned out to be one of the five actinobacterial signature proteins, and another of the five is a ParJ paralogue. We hypothesize that polar growth, which is characteristic not only of streptomycetes, but even of simple Actinobacteria, may be interlinked with ParA polymer assembly and its specific regulation by ParJ.

*Ditkowski B, Troć P, Ginda K, Donczew M, Chater KF, Zakrzewska-Czerwińska J, Jakimowicz D. Mol Microbiol. 78, 2010, 1403-1415

Laboratory of Signaling Proteins
Head: Professor Wojciech Gorczyca, Ph.D.
Acting Head since October 2010: Associate Professor Janusz Matuszyk, Ph.D.
Studies on proteins and signaling pathways involved in the activation of proinflammatory and proapoptotic transcription factors

In bacterial endotoxin (LPS)-activated macrophages, atrial natriuretic peptide (ANP) inhibits expression of proinflammatory cytokine genes (IL-1β, IL-6, TNF-α). Research in 2010 focused on the choice of cell model suitable for testing elements of the signaling pathway of ANP receptor (NPR-A), which has the activity of guanylate cyclase. Based on the results of the experiments, we selected the human monocytic leukemia THP-1 cell line for further study, since treatment of THP-1 cells with the ANP peptide led to increased levels of cyclic nucleotides (cGMP and cAMP) and protein kinase A-dependent inhibition of the LPS-induced activity of NF-κB (nuclear factor-κB).

The orphan nuclear receptor Nur77 plays an essential role in differentiation and apoptosis of different types of cells. It was found that the proximal part of the *nur77* gene promoter region has a pivotal role in activation of the *nur77* gene by nerve growth factor (NGF) in rat adrenal

pheochromocytoma (PC12) cells, while the distal part of the promoter region plays an important role in activation of the *nur77* gene in response to KCl-induced plasma membrane depolarization. A dominant negative mutant of CREB (A-CREB) inhibited the activation of the *nur77* promoter by NGF or KCl, indicating the important role of transcription factor CREB in activation of the *nur77* gene in PC12 cells, in both the response to NGF treatment and plasma membrane depolarization.

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

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

Yokenella regensburgei (Koserella trabulsii) is a Gram-negative bacterium of the *Enterobacteriaceae* family that resembles *Hafnia alvei* both biochemically and at the genetic level. *Y. regensburgei* has been isolated mainly from humans, e.g. from wounds and knee fluid, respiratory tract, urine, sputum, and from a stool. A few strains were isolated from insect intestines and from well water. *Y. regensburgei* has been implicated as an opportunistic pathogen in humans, especially under immunocompromised conditions. Most of the strains were resistant to penicillin, ampicillin, carbenicillin, colistin, and cephalothin.

The lipopolysaccharide (LPS) typically consists of O-specific polysaccharide, core oligosaccharide and lipid A. All these components are important for the biological and physical properties of the LPS molecule and take part in the pathogen-host interactions.

The structure of the core oligosaccharide segment of the *Y. regensburgei* LPS has been investigated using chemical methods, mass spectrometry and ^{1}H, ^{13}C NMR spectroscopy. It was concluded that the core oligosaccharides of the strains PCM 2476 and PCM 2477 are composed of an undecasaccharide. The combined data revealed two immunotypes of the core oligosaccharide recognized by antibodies against the whole bacterial cells. The structural differences between the core oligosaccharides are limited to the outermost terminal hexopyranose residue. In the core oligosaccharide of the strain PCM 2476 it was identified as α-D-Glc*p* and in that of the strain PCM 2477 as α-D-Gal*p*. This subtle difference between the glycoforms of the LPS core appeared to be essential for formation of the epitopes recognized by the specific antibodies directed against the *Yokenella regensburgei* whole bacterial cells. The oligosaccharides are not substituted by phosphate groups. Instead, the carboxyl groups of Kdo and galacturonic acid residues present in

the core provide the negative charges. The undecasaccharides represent a novel core type of bacterial LPS, which is characteristic for *Y. regensburgei*.

Hafnia alvei is a rare but important pathogen that is often responsible for infections of the urinary tract, respiratory tract and wounds, i.e. the most common nosocomial infections. It has been found that a multidrug resistance regulatory chromosomal locus is widespread among enteric bacteria including *Escherichia coli* and *H. alvei*. More importantly, those enteropathogenic bacteria are also capable of sharing the virulence-associated properties at the phenotypic and genetic levels, and therefore *H. alvei* should be considered as an important diarrheagenic pathogen. The serotyping scheme of *H. alvei* includes 39 O-serotypes, and preliminary chemical analyses of lipopolysaccharides isolated from 33 *H. alvei* strains have previously been reported.

NMR spectroscopy combined with chemical analysis of the O-specific polysaccharide of *H. alvei* PCM 1195 revealed the presence of glycosidically linked phosphate in addition to sugar components. The teichoic-acid-like structures among the O-antigens of *H. alvei* have been reported predominantly for some capsular polysaccharides and only rarely for the O-specific polysaccharides. Previously the phosphate was recognized as an interlinking group between the O-PS repeating units of *H. alvei* strains 744, PCM 1194, 1210 and the type strain ATCC 13337.

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

A proline-rich polypeptide complex (PRP) with immunoregulatory and procognitive activities shows beneficial effects in Alzheimer's disease. The clinical effects of orally administered Colostrinin^{TM} tablets may involve, among others, modification of cytokine release, inhibition of nitric oxide and reactive oxygen species, and amyloid β aggregation/deaggregation. Antioxidant defense includes both low molecular components and an enzymatic system comprising dismutases, catalase, glutathione reductase and glutathione peroxidase, among others. PRP and its nonapeptide component (NP) affects activities of inducible nitric oxide synthase and superoxide dismutase and reduce the level of 4-HNE. It was shown that PRP/NP can regulate the oxidative status of cells at the level of GSH-GSSG transition, influencing the GSHPx and GSSGR activity. Elevated levels of the end products of lipid peroxidation due to oxidative stress – malondialdehyde (MDA) and 4-hydroxynonenal (HNE) – were found in the brain and in ventricular cerebrospinal fluid in AD patients.

Previously an inhibitory effect of PRP on 4-HNE was observed. The present results show that PRP and NP did not affect lipid peroxidation in PBMC samples. The level of one of the oxidative stress markers, MDA, was significantly elevated in samples treated with LPS. However, no

modulatory effect was observed in the presence of PRP and NP. Sulfur-containing amino acids are easily and reversibly oxidized under relatively mild conditions. Therefore, free -SH groups were estimated as a marker of protein oxidation. Under our experimental conditions no differences in -SH group content were observed between LPS, PRP/NP and LPS + PRP/NP treated PBMC samples.

One can assume that PRP/NP, due to its inhibitory effect on HNE, SOD, and iNOS and activation of GSHPx and GSSGR but not CAT, and the fact that PRP inhibits NF-κB activity, can act as a modulatory agent of the "first line".

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

Proline-rich polypeptide complex was isolated from ovine colostrum according to the procedure elaborated in the Laboratory of General Immunochemistry. A nonapeptide fragment of the PRP complex (NP) was synthesized by a solid-phase method in the Laboratory of Chemistry and Stereochemistry of Peptides and Proteins, Faculty of Chemistry, University of Wrocław. Both PRP and NP preparations were standardized using SDS-PAGE, cytokine-inducing activity in human blood cell cultures and inhibition of NO release induced with LPS in the mouse cell line J774. To assess the effect of PRP/NP on neuritogenesis, rat PC12 Tet-On cells were used. Neuritogenesis was induced in 80% of cells treated with nerve growth factor (100 ng/ml). In the case of PRP (10 and 100 ng/ml) neuritogenesis was observed to a lesser extent and only in 30% of cells. No effect of NP was observed. It was found that both PRP and NP activate guanyl cyclase and induce cGMP in a way comparable with NGF. Preliminary results obtained with the use of soluble guanyl cyclase and nNOS inhibitors suggest, that sGC activation and induction of cGMP are not dependent on nitric oxide produced by neuronal nitric oxide synthase.

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

In 2010, studies on the interaction of nuclear proteins with the promoter region of the hFcRn gene were carried out. The aim was to determine the nature of the transcription factors binding specifically to the previously identified regulatory elements within the hFcRn promoter, in the cell lines THP1, Caco-2, Lu 106, and HUVEC. To identify nuclear proteins that interact with the hFcRn promoter, gel-supershift assays in the presence of antibodies against a given anti-transcription factor were performed.

The supershift analysis showed that:

1. transcription factors of the Sp1 family (Sp1, Sp2, Sp3), CF1/YY1, and proteins c-Fos and c-Jun participate in specific interactions with regulatory elements within the hFcRn promoter in the cell lines THP1, Caco-2, Lu 106, and HUVEC. Nuclear proteins of the Sp1 family specifically bind to regulatory sequences at positions -643, -635, -313, +82, and +251 whereas CF1/YY1 participate in binding to regulatory elements at –584 and –357. Proteins c-Fos and c-Jun, which form transcription factor AP1, bind to the site at –276,
2. transcription factor Pu1 specifically interacts only with the hFcRn promoter in THP1 cells. Pu1 binds to the sequence at position –191,
3. transcription factors AP-2α and Egr1 are involved only in specific interaction with the hFcRn promoter in HUVEC cells. AP-2α binds to the sequence at position –350, Egr1 interacts with the site at –313,
4. transcription factor NF1 binds to the sequence at position –353 of the hFcRn promoter in THP1 and Lu 106 cells.

The obtained results confirmed the earlier observations concerning the small differences with respect to the nature of the nuclear proteins binding to the hFcRn promoter in THP1, Caco-2, Lu-106 and HUVEC cells, which may point to subtle cell-type specific differences in hFcRn gene regulation.

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

Production and characterization of dromedary monoclonal antibodies recognizing DARC protein

VHHs or nanobodies are recombinant derivatives of camelid heavy-chain-only antibodies (HcAbs). Although HcAbs lack a light chain, they have a similar size and diversity repertoire as regular antibodies. VHHs, which are the domains interacting with antigen, are more stable and easier to express than the equivalent fragments of ordinary antibodies (such as Fab or scFv). DARC (Duffy antigen receptor for chemokines) is a blood group antigen, a malarial receptor for *Plasmodium vivax*, and also a receptor for CC and CXC chemokines.

A dromedary was immunized with recombinant protein consisting of the first extracellular domain of DARC and a fragment of *Staphylococcus aureus* nuclease. Lymphocytes were isolated from the dromedary's blood and cDNA was prepared. DNA fragments coding for VH (present in conventional antibodies) and VHH (present in heavy chain-only antibodies) were amplified using a suitable primer pair. DNA fragments encoding the VHHs were purified on a preparative agarose gel and re-amplified by nested PCR. The amplicons of the second PCR were ligated into a pHEN4 phagemid vector. DNA present in the plasmid encodes the VHH followed by a hemagglutinin tag, an amber stop codon and the g3 protein from M13 phage. Expression in permissive TG1 cells in the presence of helper phage M13KO7 produces bacteriophage particles that display the VHH at

their tip. In the absence of helper phage, VHH may be recovered from a periplasmic extract of TG1 cells. The library size was $7.5*10^8$. The VHH library expressed on phage particles was screened for binders to ECD1-GST constructs with wild type Fy^a and Fy^b sequences coated on ELISA plates. Antigen recognition by VHH from the periplasmic extracts of isolated clones was evaluated by ELISA. Using these methods, we obtained 27 clones with different sequences, recognizing epitopes in the first extracellular domain of DARC.

One of the VHH fragments, called CA52, was selected for further studies. Kinetics of binding of CA52 to DARC were determined by surface plasmon resonance (SPR). The epitope recognized by CA52 was determined using deletion mutants of DARC ECD1-nuclease and PEPSCAN analysis. The obtained data showed that the epitope recognized by CA52 was the pentapeptide ^{22}FEDVW^{26}. CA52 inhibits *P. vivax* invasion of erythrocytes and interleukin-8 binding to DARC. In addition, it was found that the immobilized CA52 can be used for purification of DARC from eukaryotic cells.

Carbohydrate moiety of serum IgG from psoriatic arthritis patients – changes during treatment

Immunoglobulin G contains in each heavy chain one conservative N-glycan (Asn-297); it is a biantennary oligosaccharide, poorly sialylated. Asialo forms of this glycan contain four nonreducing sugar residues: two galactoses, one bisecting GlcNAc and one proximal fucose, giving rise to 16 different glycoforms. In several diseases it is reported that the presence of galactoses in this oligosaccharide is diminished. It regards, among others, rheumatoid arthritis (RA) and psoriatic arthritis (PA), and the characteristic lack of sugars regards, in one oligosaccharide, one or both galactoses. This agalactosylation is proportional to the severity of the disease. In this investigation we analyzed 22 serum samples, taken from 11 patients with PA: samples taken before and after clinical treatment. IgG was isolated from the samples using affinity chromatography on protein G and reduced using β-mercaptoethanol. IgG samples were analyzed for the total content of galactose using gas chromatography (GC-MS), for the content of terminal Gal using ELISA test with *Ricinus communis* lectin, and for the content of terminal GlcNAc using the same test with *Griffonia simplicifolia* II lectin. Obtained results showed that in most analyzed cases serum IgG in the patients with psoriatic arthritis during clinical treatment undergoes an increase of galactosylation of its conservative N-glycans, present in the heavy chains. These data are preliminary and further experiments should be performed on more serum samples from PA patients.

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

In collaboration with Lódź Technical University we evaluated immunological activity of a series of linear and cyclic peptides. As the result, several compounds of particularly strong immunosuppressive properties were identified.

Investigating the immunoregulatory actions of lactoferrin (LF) we demonstrated its differential effects on the effector phase of the humoral and cellular immune responses in cutaneous models (responses to toluene diisocyanate and oxazolone, respectively). Several parameters, such as ear edema, vascular permeability and number of cells in the draining lymph nodes, were monitored. The results indicated that LF given intravenously before the elicitation of the cutaneous response strongly inhibited the cellular but not the humoral immune response. That result supports our earlier findings that LF inhibited activity of Th1 but not Th2 antigen-specific cells.

Studies on immunotropic properties of ubiquitin-derived peptides, done in collaboration with Wroclaw University, showed that some of them demonstrated strong immunosuppressive action on the humoral immune response *in vitro*. In addition, one peptide inhibited rejection of allogeneic skin grafts in mice.

Studies on the suppressor and anti-inflammatory actions of the cyclic tetrapeptide 4B8M were conducted in several *in vivo* murine models. The peptide inhibited the effector phase of the cutaneous, humoral response to toluene diisocyanate. Administered locally as well as systemically, it inhibited in the air pouch model the inflammatory reactions elicited by carrageenan, croton oil and zymosan. The peptide contained in an ointment inhibited non-specific skin irritations induced by salicylic acid, lauryl sulfate and trichloroethylene. In addition, the peptide suppressed ovalbumin-induced pleurisy in mice.

Bone-associated cells – fibroblasts, chondrocytes and osteoblasts are directly involved in bone building processes. The studies were focused on the ability of fibroblasts and chondrocytes to differentiate into osteoblasts. The autocrine influence of some factors expressed by these cells was studied using real-time PCR. Fibroblasts constitutively express osteopontin, osteoprotegerin, nuclear factor kappa-light-chain-enhancer of activated B cells and three isoforms of bone morphogenetic proteins: BMP-2, BMP-4 and BMP-6. These BMPs are the critical factors inducing differentiation into osteoblasts as well as chondrocytes. Chondrocytes and fibroblasts

secrete BMPs into the culture medium, which was demonstrated with Western blot analysis. These proteins are also present in their cytoplasm. Biological activity of these BMPs will be estimated in a specific biological test. The reference protein will be obtained from *Yarrowia lipolytica* after its transformation with a specific plasmid including genes for human BMPs. The construction of appropriate plasmids has already been started and the stage of gene ligation achieved.

Constitutive expression of BMP by chondrocytes could be the course of their regenerative potential. Autologous reimplantation of chondrocytes is a useful technique in surgical therapy of cartilage tissue. Human chondrocytes derived from patients with sports injuries were tested with RT-PCR and Western blot techniques for BMP as well as their receptors' expression. The obtained results indicate the presence of all investigated molecules.

Laboratory of Immunopathology

Head: Professor Irena Frydecka, M.D.

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

Comparative studies on the distribution of PB Th1, Th17 and Treg cells in patients with rheumatoid arthritis treated with MTX and TNF-α inhibitors

The balance between pro-inflammatory and anti-inflammatory T-cell subpopulations ensures homeostasis of the immune system. The chronic nature of rheumatoid arthritis (RA) strengthens the suggestion of systemic immune dysfunction, consisting in predominance of the pro-inflammatory response.

A study was undertaken to assess the state of systemic immune activity and distribution of peripheral blood (PB) Th1, Th17, and Treg cells in patients with RA before and after therapy with methotrexate (MTX) and TNF-α inhibitors (iTNF-α).

Thirty-six patients with RA and 15 healthy controls were enrolled. Sixteen patients were using MTX in a stable dose of 15 mg/weekly only (MTX group). The other 20 had exhibited an unsatisfactory response to MTX, and remained with active RA; they were qualified for treatment with iTNF-α in monotherapy or in combination with MTX (iTNF-α group). We examined CD69, CD40L, CTLA-4, IFN-gamma, IL-17, and FoxP3 expression in PB CD4+ T cells of RA patients by flow cytometry before and after the treatment for a minimum of 4-6 months. The results were compared to those obtained from healthy individuals.

Before treatment, we found markedly higher frequency of PB CD4+ T cells with expression of activation markers (CD69, CD40L) in both groups of RA patients. Among patients, the state of immune activation was significantly lower in the iTNF-α group; it was accompanied by

diminished proportions of CD4+ T cells co-expressing suppressor CTLA-4 molecule in the cytoplasm compared to the MTX group and controls. Analysis of the distribution of Th1 (CD4+IFN-gamma+), Th17 (CD4+IL-17+), and Treg (CD4+FoxP3+) cells in PB revealed a similarly increased population of Th17 in both groups of patients. Intracellular IL-17 content depended on RA activity and was significantly highest in the iTNF-α group. In RA patients, the Th17/Th1 ratio was inverted, with predominance of Th17 cells. In the iTNFα group, we observed markedly decreased both Th1 and Treg populations compared with the MTX group and controls, accompanied by impaired function of Tregs. After therapy, activation markers remained still up-regulated in all patients. However, Th17 and Treg populations as well as CTLA-4 expression were normalized. In the MTX group, increase in spontaneous production of IFN-gamma in CD4+T cells was found. The iTNF-α group remained with a significantly lower Th1 population compared to the MTX group and controls.

The study confirms expansion of the Th17 subset in active RA. Even after therapy, RA patients remained with a partial state of immune activation. Progression of RA results in gradual exhaustion of both activating and inhibitory potential of PB CD4+ T cells as well as a systemic imbalance of Th1, Th17, and Treg cells, of which Th1 defects seem to be irreversible.

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

Laboratory of Tumor Molecular Immunobiology
Head: Professor Leon Strządała, Ph.D.

Activation of CD47 induces non-apoptotic death of ras-transformed fibroblasts

The negative impact of thrombospondin-1 (TSP-1) on endothelial cells, which consists of proapoptotic, antimigratory, and antiproliferative effects, is largely attributed to the ligation of the CD36 receptor via the region of the molecule containing the type 1 repeats (TSR). Although the antitumor activity of CD36-directed peptides has been encouraging, their effects seem ostensibly less dramatic than might be expected from the impact exerted by the corresponding full-length molecule. This raises a possibility that other TSP-1 domains and functions could possess hitherto unappreciated anticancer properties. TSP-1 is known to interact with the CD47 receptor expressed on the surface of various cells, and in a manner that has no known antiangiogenic consequences. This binding is dependent on the C-terminal domain of TSP-1, from which functional peptide analogues (4N1K) have been recently derived and characterized. CD47 ligation was previously described to induce an atypical cell-death program, for example in CD-3/Ras activated T lymphocytes, which combines some biochemical features of apoptosis and autophagy.

To determine whether the nature of the CD47-dependent demise of Ras-expressing cancer cells resembles these events, we examined the properties of mouse dermal fibroblastic cells harboring mutant H-ras (B6ras line) as they were dying under the influence of the CD47-directed agents. Indeed, we observed that treatment with anti-CD47 antibody or 4N1K peptide resulted in a decline in cell numbers, and an increase in staining with Annexin V, but without a proportional uptake of propidium iodide. Moreover, whereas treatment of these cells with etoposide induced caspase 3 activation and other features of apoptosis, they were not observed in the same cells subjected to death-inducing treatment with 4N1K. CD47-mediated cell death was previously associated with features of autophagy, a process of partial autolysis which may result in either increase or decrease in cancer cell survival. Some of the best characterized properties associated with autophagy are the increased formation of acidic cellular vacuoles, detectable by staining with acridine orange, and LC3 punctation. Interestingly, B6ras cells exposed to 4N1K peptide stained positively for both of these markers. Altogether, our results showed that CD47 activation causes atypical cell death of Ras-expressing cancer cells, which may contribute to antitumor activity of TSP-1.

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

Mechanism of antitumor activity of new genistein analogs

The estrogenic activity of the new genistein derivatives was studied. We showed that derivatives of genistein in a low concentration (less than 1 uM) did not stimulate the proliferation of MCF-7 cells (in contrast to previously published data). What is more, we observed that the analogs of genistein IFG-027 and IFG-043 as well as the complex of genistein with xyloglucan reduced the expression of estrogen receptors alpha and beta. We suppose that these compounds could act as antagonists of ER, which resulted in their anti-estrogenic activity. The second genistein complex, schizophyllan x genistein, increased the expression of estrogen receptors alpha and beta. This result could indicate its agonist nature toward the estrogen receptors and estrogenic activity.

Estimation of cytolytic lymphocyte activity in MC38 tumor-bearing mice induced by cellular vaccines based on tumor or dendritic cells

Tumor or dendritic cells (DCs) were used as adjuvants supporting 5-fluorouracil (5FU) or cyclophosphamide (CY) activity. The peritumoral administration of DCs stimulated with tumor cell lysate (DC/TAg), naive DCs (DC naive) or their combination with IL-2 producing tumor cells (MC38IL-2) induced immunity against syngeneic tumor. The main effect of combined therapy was dependent on the

type of cytostatic as well as the capability of particular vaccines. The obtained results pointed to higher efficacy of CY-based combined therapy compared with 5FU-based therapy due to the increase in percentage of $CD8^+$, $CD49b^+$, and $CD4^+$ cells among splenocytes harvested 7 days after the last vaccine administration.

Dendritic cells genetically modified to produce IL-2 were also used in combined chemo-immunotherapy of mice with transplantable tumor. Within the long-term observation the effect of CY as well as 5FU administration accompanied by the cellular vaccines was performed. The application of combined therapy with cytostatics caused decay of the tumor tissue structure, associated with more intensive influx of $CD4^+$ than $CD8^+$ T cells. $CD4^+$ T cells were preferentially localized in connective tissue while $CD8^+$ cells were visualized in necrotic areas of tumor tissue. However, higher numbers of both $CD4^+$ and $CD8^+$ cells were observed after the use of CY combination with DCs, especially along with MC38/IL-2. Administration of 5FU followed by cellular vaccine resulted in markedly lower influx.

Studies on an efficient carrier for siRNA delivery

In 2010 studies on an efficient carrier for siRNA delivery were conducted. Experiments were performed in a mouse melanoma B16 model, using siRNA directed against integrin β3. Studies on linear polyethylenimine were performed with a special focus on chemical modifications of PEI chains. Furthermore, the transfection efficiency of polyethylenimine combined with hydrophobic molecules was examined. These included PEG, cyclodextrins and oxazolines. In some cases, an increase in the efficiency of transfection was observed as compared to commercially available PEIs.

Finally, the usefulness of low molecular weight proteins (e.g. protamines) as transfection agents was studied. In this case, no efficient transfer of siRNA to the B16 cell was observed.

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

Studies on methotrexate-fibrinogen conjugates

The Laboratory of Biomedical Chemistry is focused on the development of drug-carrier conjugates for the treatment of experimental cancer and immunological diseases. We investigate the biochemical properties and biological activities of protein (fibrinogen, albumin, antibodies) and carbohydrate (glucose or mannose polymers) methotrexate and raltitrexed conjugates.

Physiochemical studies of bacteriophages

Besides the chemical modification of macromolecules, we are investigating the physicochemical properties of bacterial viruses, or bacteriophages. In particular, we aim to develop an effective procedure for the purification of bacterial viruses.

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

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

Immunogenetics of human diseases

(a) Association of cryptorchidism in prepubertal boys with DRB1*11

Cryptorchidism has been a frequent syndrome occurring in 1-2% of males within the first year of age. Autoimmune reactions, particularly directed to testicular elements and/or spermatozoa, have been found to be often associated with cryptorchidism. Therefore we investigated the frequency of HLA class II alleles in order to recognize possible genetic predisposition for cryptorchidism in prepubertal boys in the Caucasoid population. Sixty prepubertal boys with cryptorchidism and 60 healthy boys were examined for their *HLA-DR* and *-DQ* types using DNA obtained from peripheral blood cells. The typing of *HLA-DR* and *-DQ* was performed by using the PCR-SSP low resolution method.

Overall, in comparison to the control healthy population, in prepubertal boys with cryptorchidism a trend for lower representation of *HLA-DRB1*08* and *HLA-DQB1*04* was observed. An interesting *post hoc* finding indicated in boys with cryptorchidism and history of infertility in the family the highest statistical difference in *HLA-DRB1*11* frequency ($p = 0.0002$, *p corrected* = 0.0006, odds ratio 8.167) when compared with healthy boys. Additionally, prepubertal boys with bilateral cryptorchidism and unilateral cryptorchidism differed in allelic frequencies from controls for *HLA-DRB1*11* and for *HLA-DRB1*08*, respectively.

Conclusions: Statistically significant differences were found between pre-pubertal boys with cryptorchidism with respect to *HLA-DRB1*08* and *HLA-DQB1*04* allelic frequencies. The strong association of cryptorchidism and history of infertility in a family with *HLA-DRB1*11* suggests that familial and sporadic cryptorchidism may present distinct genetic backgrounds.

(b) Genetic factors contributing to high risk of neoplastic disease: study on HMGB1 gene polymorphisms in ovarian cancer

The *HMGB1* gene belongs to a family of high mobility group (*HMG*)-box genes. It encodes a nucleoprotein expressed in all cell types and localized in chromatin. HMGB1 participates in DNA

repair inside the cell, but also acts as a proinflammatory cytokine extracellularly. We studied several *HMGB1* single nucleotide polymorphisms (SNPs) and determined *HMGB1* haplotypes in patients with ovarian cancer and in control women. Genotypes of all SNPs were in Hardy-Weinberg equilibrium, except for one SNP which was underrepresented in heterozygous form in patients. Three frequent haplotypes and three rare ones were observed in our Polish population sample. Combinations of these haplotypes gave five frequent (8-32%) and several rare (3% at most) diplotypes whose distribution between patients and controls did not differ significantly. Eight out of 198 patients exhibited allergic reactions; half of them represented one homozygous diplotype. Three diplotypes seemed to be potentially associated with toxicity of cis-platinum for the digestive and nervous systems.

Results of grant activities

Association of KIR and HLA genotype with atopic dermatitis

Atopic dermatitis (AD) is one of the most common skin diseases. It is a multifactorial condition with a complex genetic background, and only some genes contributing to susceptibility to AD are known. We are studying a possible role of killer immunoglobulin-like receptor genes (*KIR*) in AD, which has not been tested yet for these genes. KIR molecules are expressed on natural killer (NK) cells and subpopulations of T lymphocytes. The *KIR* gene complex is characterized by a haplotype polymorphism, i.e., differences in distribution of particular *KIR* genes between unrelated individuals. *KIR* genotype affects susceptibility to a panel of human diseases. In all, 248 patients diagnosed with AD and 690 unrelated healthy control individuals were typed for *KIR* gene presence or absence. Frequencies of the great majority of *KIR* genes did not differ between patients and controls. The only exception was *KIR2DS1*, whose frequency was significantly ($p = 0.0005$) lower in patients than in controls, and this difference persisted even after Bonferroni correction. This result suggests a protective effect of KIR2DS1 molecule presence on NK or T cells in AD.

Does a pregnancy-derived microchimerism contribute to pathophysiology of psoriasis?

Pregnancy is one of the main causes of natural microchimerism (the stable presence of a small number of genetically distinct cells in the organism). Microchimerism is caused by the passage of fetal cells via the placenta into the mother's circulation or vice versa. It does not cease after delivery and may persist for years. Microchimeric cells are detected mostly among hematopoietic stem cells defined as CD34+ or CD34+CD38+, but may be present also within T or B lymphocyte subpopulations or monocytes. A question arises whether semiallogeneic fetal microchimeric cells

present in the mother might be involved in pathogenesis of some autoimmune diseases such as psoriasis. We attempted to answer this by HLA-DRB1 allele typing of CD34-enriched and CD34-negative peripheral blood mononuclear cells (PBMC). Fifty-one patients with psoriasis vulgaris (31 females and 20 males) were enrolled in the study. Microchimerism was found both in patients and controls. The differences between the patient group and controls were not significant. The frequency of microchimerism was slightly higher in controls compared to psoriatic patients (relative frequency in the whole population – 0.32 and 0.23, respectively, $p = 0.57$). In positive samples microchimeric cells were found in both CD34+ and CD34⁻ cells, in CD34+ only or in CD34⁻ only. The differences in the frequency of microchimerism in CD34+/CD34⁻ cells and in CD34+ only cells between patients and controls were not significant. However, in patients, in contrast to the controls, we found microchimeric cells in the CD34⁻ population, and this difference in the frequency of microchimerism observed only in the CD34⁻ compartment was highly significant ($p < 0.002$). To the best of our knowledge this study presents for the first time the presence of microchimerism in psoriasis. Our data may suggest that in some patients with psoriasis microchimeric cells represent an immunologically active PBMC in contrast to healthy controls, which may be the effect of differentiation of microchimeric progenitor CD34+ stem cells into functional CD34⁻ leukocytes, as postulated before.

MDR1 gene polymorphisms and therapy efficiency in ovarian cancer

Frequencies of *MDR1* gene polymorphisms were tested in 198 patients with ovarian cancer and in 263 healthy individuals (146 females and 117 males). Similarly to other Caucasian populations, there were three common haplotypes (11-43%) and several rare ones (below 5%), making several diplotypes. No differences between patients and controls in distribution of diplotypes were observed. However, some diplotypes seemed to associate with response to therapy.

Laboratory of Clinical Immunology
Head: Professor Andrzej Lange, M.D.
Genetic background and pathomorphological evaluation of allogeneic reaction after transplantation of hematopoietic stem cells

1. *Analysis of Th17 cells in relation to post-transplant complications: Th17 CD4+ cells disappear from blood at the onset of acute GvHD*

The proportions of IL-17 producing cells (in 7-day intervals beginning from hematological recovery covering the time of risk of aGvHD), serum CRP levels and NOD2/CARD15 gene

mutations were analyzed in relation to GvHD complications and viral reactivations in 58 patients undergoing allogeneic HSCT.

It was found that: Apparent clinical manifestation of aGvHD was associated with a low proportion of Th17 lymphocytes in blood, which declined in blood during 3 to 7 days prior to overt aGvHD. Gut manifestation was associated with a significantly lower number of blood Th17+ lymphocytes than seen in patients with only skin lesions. Moreover, the presence of the NOD2/CARD15 mutation may constitute a risk factor of gut aGvHD as all 3 patients with gut manifestation and investigated for NOD2/CARD15 mutation had this gene mutated, which was seen only in 4 out of 17 patients with skin symptoms.

No correlation was observed between CRP elevation and the proportion of IL-17 producing cells in CD4+ lymphocytes.

2. Analysis of subpopulations of CD4 lymphocytes: Acute GvHD patients have low proportions of naïve and terminally differentiated memory CD4+ lymphocytes

Naïve (CD45RA+, CCR7+, CD4+), central memory (CD45RA-, CCR7+, CD4+), effector memory (CD45RA-, CCR7-, CD4+) and terminally differentiated memory (CD4+, CD45RA+, CCR7-) T-cell populations were studied in alloHSCT patients on the day of hematological recovery and 4 weeks after HSCT. Clinical outcome observation included the manifestation of aGvHD and viral reactivations. Naive T cells were in higher proportions in patients lacking than in those having aGvHD at the beginning of hematological recovery and 4 weeks later. TEMRA cells were higher in aGvHD patients at two time points at the beginning of hematological recovery and four weeks later. There were no differences when blood central and effector memory CD4+ cells were analyzed. Therefore, aGvHD patients were handicapped with respect to the number of naïve and TEMRA CD4+ lymphocytes in the blood. This likely reflects an impairment of thymus function and consequently lymphocyte differentiation into effector cells in aGvHD patients.

3. Analysis of risk factors of viral reactivation in patients after HSCT:
Risk factors of CMV, HHV6 and EBV reactivation after allogeneic hematopoietic stem cell transplantation

One hundred and two (45 SIB, 57 MUD) HSCT recipients were followed for immunological reconstitution, post-transplant complications and CMV, HHV6 and EBV reactivations. Numbers of herpes viral DNA copies in peripheral blood cells were determined in patients within the observation period ranging from day 7 to one year post HSCT. Numbers of EBV and CMV copies ≥100/10^5 cells appearing at least on one occasion were significantly associated with post-transplant complications including aGvHD and relapse. CMV, EBV and HHV6 DNA copies were

detected in 34%, 27% and 26% of patients, respectively. It was found that CMV reactivation was more frequent in patients: with aGvHD (grade >=I), receiving an HLA partially matched graft, who were adults as compared to children, having a low proportion of CD8high+ CMV pp65 reactive cells, or with CMV IgG seropositivity. EBV reactivation was associated with: encephalitis, female recipient and donor gender, receiving HSCT from EBV IgG seropositive donors, and having a low proportion of CD8high+ EBV BLMF-1 and LMP-2 reactive cells. HHV6 reactivation was more frequent in patients: receiving a myeloablative conditioning regimen, with encephalitis, receiving an HLA matched graft, or lacking extensive cGvHD. The multivariate logistic regression analysis documented that recipient age over 16 years, HLA partial mismatch, aGvHD (grade>I) and recipient CMV IgG seropositivity are independent risk factors of CMV reactivation. Female donor and recipient gender are risk factors for EBV reactivation, and male donor gender, myeloablative conditioning regimen, transplantation from MUD donors, HLA match, and lack of extensive cGvHD are independent risk factors of HHV6 reactivation.

Patients with HHV6 reactivation had low numbers of CD4+ effector memory lymphocytes in the blood, which was associated with rather poor survival

The aim of this study was to investigate reconstitution of subpopulations of CD4+ lymphocytes in the context of β-herpes viruses reactivation shortly after HSCT. There were 2 observation points, at the beginning of lymphocyte recovery and 3 weeks later. CD4+ T cell subsets were defined as follows: naive cells CD4+CCR7+CD45RA+ (N), central memory CD4+CCR7+CD45- (CM), effector memory CD4+CCR7-CD45+ (EM), and terminally differentiated memory cells CD4+CCR7-CD45RA+ (TEMRA). CMV, EBV and HHV-6 DNA copies were detected in blood with the use of QT-PCR in one-week intervals. It was found that the CD4+ lymphocyte count was lower in patients having CMV copies as compared to those having EBV and/or HHV6 copies at the beginning of lymphocyte recovery, and EM cells behaved similarly. EM cells were seen in patients having CMV reactivation and in those with EBV and/or HHV-6 reactivation, respectively. The situation changed three weeks later; it appeared that patients having HHV6 reactivation any time during the observation period had lower CD4+ lymphocyte numbers as compared to patients lacking β-herpes viruses reactivations and to those with CMV and EBV reactivation. Again EM cells contributed greatly to these differences. EM CD4+ lymphocytes were 41/ul, 105/ul, 80/ul and 160/ul in patients having HHV-6, CMV or EBV and lacking beta-herpes viruses reactivation, respectively. Patients with HHV6 had lower number of EM CD4+ lymphocytes as compared to patients lacking ß-herpes viruses reactivations. It appears that HHV6 is the main player in shaping lymphocyte reconstitution post transplant, affecting predominantly EM cells, which influences the outcome of transplantation. In this group

of patients probability of one year survival was 50% vs. 65% in patients having and lacking HHV6, respectively.

4. Analysis of the association between non-classical HLA (HLA-E and HLA-G locus) and the outcome of allogeneic hematopoietic stem cell transplantation

The present study aimed to determine whether typing for non-classical HLA (HLA-E and HLA-G locus) alleles may be of prognostic value for the outcome of allogeneic hematopoietic stem cell transplantation (HSCT) and optimize standard donor-recipient matching for classical HLA loci. One hundred patients and their donors were typed for the presence of two HLA-E alleles (*0101, *0103) coding expressed HLA-E molecules and the HLA-G ins/del polymorphism. Acute GvHD was more frequently observed after HLA-E mismatched transplants being matched at the high resolution level for 5 classical HLA loci (HLA-A, B, C, DRB1 and DQB1; 10/10 alleles). Fatal complications were more frequent in patients who presented with acute GvHD complications. Recipient HLA-E*0103 homozygosity was associated with improved likelihood of patient 3 yrs overall survival. This tendency was also seen in patients transplanted with donors carrying the HLA-G del/del genotype. Both these factors, the presence of HLA-E*0103, 0103 in the patients and/or HLA-G del/del in the donors, were found to significantly improve survival. These results suggest that typing of non-classical HLA-E and G alleles and non-HLA genes (*HSP70-hom* alleles), in addition to standard donor-recipient matching for 5 classical HLA loci, is of prognostic value for the outcome of allogeneic HSCT.

DEPARTMENT OF MEDICAL IMMUNOLOGY
Head: Professor Jacek C. Szepietowski, M.D.

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

The role of T lymphocytes in the process of angiogenesis of ectopic tissue in women with endometriosis

In women with endometriosis T lymphocytes stimulated with IL-2 produced increased amounts of bFGF and eotaxin in comparison with T lymphocytes of fertile women with ovarian cysts (used as a control group). In fertile women with ovarian cysts in contrast to women with endometriosis the supernatants from culture of stimulated T lymphocytes inhibited the growth of human umbilical vein endothelial cells (HUVEC). These observations suggest that T lymphocytes in fertile women may be involved in the control of the process of angiogenesis during accidental implantation of ectopic endometrial tissue.

The involvement of antigen-presenting cells in establishing peripheral tolerance during the preimplantation period of pregnancy in mice. Supported by Gemini Cost Action FA0702

The aim of this study was to assess the expression of co-stimulatory molecules on spleen antigen-presenting cells (APCs) in mouse during the preimplantation period of pregnancy. At 0.5 day after mating expression of a few costimulatory molecules on APCs was significantly changed. In contrast, 3.5 days after mating all examined APCs showed significantly higher expression of CD86 molecule. CD11b(high) and F4/80+ cells showed increased levels of CD40 antigen in comparison with pseudopregnant mice. However, the number of CD11c+CD80+ cells decreased at both 0.5 and 3.5 days after mating. In conclusion, during the preimplantation period of pregnancy the costimulatory phenotype of peripheral APCs is changed. The increased expression of CD86 on all examined APCs and decreased number of dendritic cells with CD80 molecule expression suggests broad involvement of an activating costimulatory pathway dependent on CD28 molecules present on lymphocytes.

The development of lab-on-chip with optical detection- diagnostic device for quick and cheap quality qualification of bovine embryos. Micro- and Nano-Systems in Chemistry and Biomedical Diagnostics. Supported by POIG 01.03.01-00-014/08-00 2009-2012

The aim of this study was to evaluate the usefulness of a micro-flow "lab-on-chip" device for supravital evaluation of apoptosis in mouse embryos. Embryos were obtained at 2.5 days of pregnancy and cultured for 10-12 hours in the presence of actinomycin D. After the culture, the embryos were morphologically assessed and stained with annexin V conjugated with fluorescein. The embryos were placed in a silicon-glass chip in the measuring cell integrated with optical fibers that supply excitation light and drain emitted light. The intensity of fluorescence of each single embryo was examined. After measurement, the embryos were cultured for the next 24 hours, and morphologically re-evaluated. Significant differences in fluorescence intensity between the control and experimental (apoptosis-induced) group were observed. In addition, neither the staining procedure nor chip evaluation of embryos affected their *in vitro* development to the blastocyst stage.

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

Endothelial precursor cells

The aim of the work was to determine phenotypic and functional characteristics of endothelial precursor cells. We used a panel of cell-specific markers identifying these cells at successive

stages of their multiplication and differentiation towards functional endothelial cells. Particularly interesting are markers connected with the expression of multidrug resistance phenotype, particularly those present during tumor neoangiogenesis. Using techniques of isolation of human CD133 positive cells from cord blood, we established two unique cell lines of early endothelial precursor cells. Their characteristics including phenotypic and functional characteristics in *in vitro* conditions were summarized in two original papers: one has already been published, and the second is in preparation. These cell lines were also submitted for the international patenting procedures.

The abovementioned studies are performed within the framework of Polish-French collaboration with the laboratory of Dr Claudine Kieda from Centre Biophysique Moleculaire CNRS, Orleans, France, and with the group of Prof. Józef Dulak from the Department of Medical Biotechnology of Jagiellonian University in Kraków.

The study was performed in collaboration with clinics of Wrocław Medical University concerning the levels of circulating endothelial precursor cells in endometrial cancer and in psoriasis. The results are evaluated in the context of clinical parameters of the patients, as potential disease markers.

The role of mucin in endothelial cell – breast carcinoma cell adhesive interactions

In breast carcinoma cells the phenomenon of incomplete synthesis of O-glycans and appearance of tumor-specific Tn and sialyl-Tn as well as T and sialyl-T antigens are the result of changes in the expression of some specific enzymes – C2GnT1 and ST3Gal-I glycosyltransferases. As we previously demonstrated on mouse mammary carcinoma MDA-MB-231 cell line transfected with MUC1 cDNA, the expression of both glycosyltransferases depends on the MUC1 mucin expression level. Its overexpression in mouse, but also in human breast carcinoma cell lines, was accompanied by an increase in T and sialyl-T antigen levels, with simultaneous, almost one hundred-fold decrease in C2GnT1 and ST3Gal-I glycosyltransferase levels. To confirm the observation, we prepared two non-functional human breast carcinoma cell lines with blocked MUC1 synthesis, using the strategy of iRNA. The results obtained confirmed that MUC1 is involved in regulation of the C2GnT1 glycosyltransferase gene. To examine the mechanism of regulation, the C2GnT1 promoter was cloned into a pGL3 vector, and its activity was analyzed in human breast carcinoma cell lines, parental and those with MUC1 overexpression/suppression. The results obtained showed no direct relationship between MUC1 level and C2GnT1 promoter activity, indicating a different, post-transcriptional regulation level. Further experiments, blocking microRNA synthesis with actinomycin D, suggested that regulation of the C2GnT1 gene takes place at the posttranscriptional level.

Publications – 2010

Articles published in the journals from Thomson Reuters Master Journal List

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