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Research Report 2006

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

Laboratory of the Molecular Biology of Microorganisms

Head: Professor Jolanta Zakrzewska-Czerwińska, Ph.D.

The molecular basis of replication and gene expression and the design of compounds inhibiting these processes

Replisome localization in vegetative and aerial hyphae of Streptomyces coelicolor: asynchronous replication within the multinucleoid organism

Streptomycetes, Gram-positive soil bacteria known for their ability to produce many valuable antibiotics and other secondary metabolites, are among the most striking examples of multicellular bacteria. *Streptomyces* colonies consist of a branched vegetative mycelium bearing aerial hyphae that form long chains of exospores. Both vegetative and rapidly growing aerial hyphae are multigenomic, with the latter containing up to 50 uncondensed copies of the linear chromosome in one tip compartment. As an aerial hyphae stops growing, the chromosomes condense and are segregated into unigenomic pre-spore compartments which metamorphose into chains of easily separated spores.

We examine for the first time the subcellular localization of the replisome machinery within multinucleoid compartments of the vegetative and aerial hyphae of *S. coelicolor* using a functional fusion of DnaN (β-subunit of DNA polymerase III) to enhanced GFP. Our results showed that chromosome replication takes place along a large portion of the vegetative and aerial hyphae. The replication dynamics appear to depend on growth rate, since the apical compartments of the aerial mycelium have more active replication than any other compartments. Within a single compartment, the number of "current" ongoing DNA replications was lower than the expected chromosome number, and the appearance of fluorescent foci was often heterogeneous, indicating that this process is asynchronous within compartments and that only selected chromosomes undergo replication. Thus, replication appears to follow the Jesuit dictum “many are called, but few are chosen”.

Laboratory of Signaling Proteins

Head: Associate Professor Wojciech Gorczyca, Ph.D.

Studies on proteins involved in the activation of proinflammatory transcription factors in immune cells

The aim of our studies was to determine the role of the cGMP-dependent signaling pathways in inflammatory rat macrophages and neutrophils. Although it is known that cGMP modulates the inflammatory response of macrophages, the underlying molecular mechanisms remain poorly understood. Looking for proteins regulated by cGMP in rat peritoneal macrophages (PMs), we have analyzed the expressions and activities of cGMP-hydrolyzing and cGMP-regulated phosphodiesterases (PDEs). In freshly isolated peritoneal exudate macrophages (PEMs), enzymes belonging to families PDE1-3, PDE5, PDE10, and PDE11 were detected. Analysis of the substrate's specificity, sensitivity to inhibitors, and subcellular localization showed that PDE2 and PDE3 are the main cGMP-regulated PDEs in PEMs. The profile of PDE expression was altered by maintaining PEMs in culture and treatment with bacterial endotoxin (LPS). After 24 h of culture, the levels of PDE2, PDE3, and PDE11 were markedly decreased. However, their expression and activity recovered after treatment of the cultured cells with LPS. A similar pattern of change was observed in the expression of TNFα, but not in guanylyl cyclase A (GC-A). LPS also up-regulated PDE expression in resident peritoneal macrophages (RPMs), although not all PDEs present in PEMs were detected in RPMs. Taken together, our results showed that in rat PMs the expression of cGMP-dependent PDEs positively correlated with the activation state of the cells. Studying rat neutrophils, we found that cells isolated from rats treated intraperitoneally with peptone protease cannot use the nitric oxide/soluble guanylyl cyclase/cGMP-dependent protein kinase (NO/sGC/PKG) signaling pathway, which has been reported to affect important functions of circulating neutrophils. Although PKG was detected at both the mRNA and protein levels in peripheral blood neutrophils (PBNs) of control rats, it was expressed neither in PBNs nor in peritoneal exudate neutrophils (PENs) of provoked rats. Also, mRNA of the α and β chains of heterodimeric sGC was present in PBNs, but absent in PENs. PBNs accordingly responded to activators of sGC with cGMP synthesis, while PENs did not. These results showed that neutrophils recruited by a provoking agent lost PKG and, in the case of PENs, also sGC and thus the capacity to respond to NO with cGMP signaling. Such downregulation of the sGC/PKG pathway is likely a result of the high activity of inducible NO synthase observed in inflammatory neutrophils.

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

Plesiomonas shigelloides is a Gram-negative rod associated with episodes of intestinal infection and outbreaks of diarrhea in humans. The extraintestinal infections caused by this bacterium, e.g. endopthalmitis, meningitidis, bacteremia, and septicemia, usually have gastrointestinal origin, serious course, and high fatality rate. Lipopolysaccharide (LPS, endotoxin) is important as virulence factor in the enteropathogenicity of this bacterium. LPS, built of a O-specific chain, core oligosaccharide, and lipid A, is the main constituent of the outer membrane of Gram-negative bacteria. LPS plays an important role in the effective barrier properties of the outer membrane. It constitutes a "pathogen-associated molecular pattern" for host infection by Gram-negative bacteria and is one of the most powerful natural activators of the innate immune system. LPS plays a key role during severe Gram-negative infections, sepsis, and septic shock. Lipid A is an immunomodulatory center of endotoxin and is recognized by different classes of receptors, including Toll-like receptors.

Lipopolysaccharides of *P. shigelloides*, especially their lipid A part, have not been extensively investigated. We have determined the structure of the entire molecule of LPS isolated from serotype O74 and completed the structural investigation of serotype O54 endotoxin. The lipopolysaccharide of *P. s shigelloides* serotype O74 was obtained by the hot phenol/water method, but unlike most S-type enterobacterial lipopolysaccharides, the O-antigens were preferentially extracted into the phenol phase. The poly- and oligosaccharides released by mild acid hydrolysis of the lipopolysaccharide from both the phenol and water phases were separated and investigated by ^{1}H and ^{13}C NMR spectroscopy, MALDI-TOF mass spectrometry, and sugar and methylation analysis. The O-specific polysaccharide and oligosaccharides consisting of a core, the core with one repeating unit, and the core with two repeating units were isolated. It was concluded that the O-specific polysaccharide is composed of a trisaccharide repeating unit with the following structure: [→2)-b-D-Qui*p*3NAcyl-(1→3)-a-L-Rha*p*2OAc-(1→3)-a-D-Fuc*p*NAc-(1→], in which D-Qui3NAcyl is 3-amino-3,6-dideoxy-D-glucose acylated with 3-hydroxy-2,3-dimethyl-5-oxo-pyrrolidine-2-carboxylic acid. The major oligosaccharide consisted of a single repeating unit and a core

oligosaccharide. This undecasaccharide contains information on the biological repeating unit and on the type and position of the linkage between the O-specific chain and the core. The core oligosaccharide was composed of a non-phosphorylated octasaccharide, which represents a novel core type of *P. shigelloides* LPS characteristic for serotype O74. The similarity of the isolated O-specific polysaccharide with that found on intact bacterial cells and lipopolysaccharide was confirmed by HR-MAS NMR experiments. The characteristic feature of the core decasaccharide isolated by mild acid hydrolysis of *P. shigelloides* O54 LPS is the lack of phosphate groups and the presence of GalA. The lipid A of *P. shigelloides* O54 is a novel structure. Primary-linked fatty acids are identical to those present in *Neisseria spp.* lipid A, but contrary to *Neisseria spp.*, they are asymmetrically distributed (2 + 4). Thus *P. shigelloides* O54 lipid A belongs to the group of *E. coli*-type lipid A. On the basis of the phosphorylation of GlcN disaccharide at O-1 and O-4' and the length of the asymmetrically distributed acyl groups limited to 12 or 14 carbons, the high cytokine-inducing activity of *P. shigelloides* O54 LPS was expected and confirmed by the experimental data. The lipid A isolated both from phenol- and water-soluble *P. shigelloides* O74 LPS is heterogeneous and is represented by three forms differing in the acylation pattern. The main population of lipid A isolated from phenol-soluble LPS contained an unsaturated fatty acid, 9*c*-16:1. It is a rather uncommon constituent of lipid A molecules, and to date it was only found in lipopolysaccharides isolated from pathogenic *Yersinia* species grown at low temperature. Palmitoleate is also present in lipid A of *E. coli* and *S. typhimurium* cells subjected to low temperature. This unsaturated fatty acid was not present in lipid A of *E. coli* grown at 37°C. It was found earlier that palmitoleoyl transferase was induced upon cold shock. *P. shigelloides* O74 cells cultured at 37°C produced lipid A, containing 9*c*-16:1. This therefore suggests that this specific palmitoleoyl transferase is also active in these bacteria at elevated temperature. The difference between *P. shigelloides* and *E. coli* LPS in the “general structure” of their lipid A and core parts did not affect the activity of *P. shigelloides* endotoxin in *in vitro* cytokine induction assays. The presence of GalA and absence of phosphate residues in the core oligosaccharide of S-type *P. shigelloides* O54 LPS could play a role in disaggregation, less effective neutralization, and clearance mechanisms resulting in the higher toxicity of this LPS *in vivo*.

Laboratory of General Immunochemistry

Head: Associate Professor Maria Janusz, Ph.D.

Studies on the mechanism of action of a proline-rich polypeptide complex (PRP): influence on β-amyloid aggregation

Extracellular deposits in the brains of Alzheimer's disease (AD) patients are formed by insoluble aggregates of amyloid β peptides Aβ 1-40 and Aβ 1-42. It is possible that beneficial clinical effect of the proline-rich polypeptide complex (PRP/Colostrinin) in AD patients is connected with an influence of its peptide components on the aggregation of Aβ peptides. Many functions of the PRP complex, such as regulation of immune response in mice and cytokine and reactive oxygen species induction in human blood cells, can be replaced by one of its components: the nonapeptide Val-Glu-Ser-Tyr-Val-Pro-Leu-Phe-Pro (NP). Aggregates formed during 1-6 days of incubation of $A\beta_{42}$ in the presence or absence of NP were analyzed with the use of electron microscopy (EM) and atomic force microscopy (AFM). Incubation for several days at 37°C gradually induces aggregation and fibryl formation in $A\beta_{42}$ solutions (EM). After six days, thickly packed structures of 15-52 nm were observed in AFM. In the presence of NP, no fibrillation of Aβ was observed in EM, and only small numbers of structures with a height not exceeding 8.5 nm were observed in AFM.

During aggregation (six days incubation at 37°C), the circular dichroism (CD) spectrum indicated the formation of the β structure, with a minimum at 213-216 nm and a maximum at 193-195 nm. When $A\beta_{42}$ was incubated in the presence of NP, the CD spectrum was similar to that of freshly prepared samples and was characteristic of an aperiodic structure. The inhibitory effect of NP on the aggregation of Aβ was also shown with Thoflavin T, an indicator of the formation of the β structure.

The mechanism of the formation of Aβ aggregates in the brain is not fully understood. There is increasing evidence that metal ions, especially Ca^{2+} ions, may facilitate the formation of aggregates. We have shown, using THP1 and HL60 cells lines as a monocytic/macrophage model, that PRP attenuates the increase in the intracellular concentration of Ca^{2+} ions $[Ca^{2+}]_i$ induced by proinflammatory activators, such as FMLP or $A\beta_{42}$. The results of our studies on the effect of PRP peptides on the aggregation of Aβ 1-42 and on $[Ca^{2+}]_i$ might, in part, explain the therapeutic effect of PRP/Colostrinin in AD patients.

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

We investigated the construction of recombinant, tetrameric Fab fragment containing an acceptor peptide for biotinylation. The *Escherichia coli* biotin holoenzyme synthetase (BirA) catalyzes the transfer of the biotin to the epsilon-amine group of a specific lysine residue of the biotin carboxyl carrier protein (BCCP) subunit of acetyl-CoA carboxylase. We constructed the vector encoding the biotinylation acceptor sequence by introducing cDNA encoding the optimal acceptor sequence into the pComb3H plasmid. To evaluate the vector, we used the NNA7 (anti-glycophorin A type N antibody) Fab fragment previously obtained by light-chain shuffling of a monoclonal antibody, N92. The purified NNA7 fragments were then analyzed by SDS-PAGE, ELISA, and agglutination assay. After reaction with the biotin ligase *in vitro*, these Fab fragments became biotinylated and could be used in such assays as ELISA or Western Blotting using avidin-alkaline phosphatase conjugate as a detecting reagent. After mixing with avidin, the agglutination power of the anti-glycophorin Fab fragments increased fourfold.

We performed pilot experiments on the use of Dynabeads® Talon™ beads for the purification of recombinant Duffy proteins containing histidyl tags. These beads are uniform, paramagnetic polystyrene beads composed of a metal chelator which complexes the cobalt ions. The imidazole rings of histidine residues present in a polyhistidine peptide fragment are able to interact with the cobalt ions, resulting in a protein binding to the beads with enhanced selectivity. The Duffy proteins containing histidyl tags were expressed in the K562 erythroleukemia cell line and were purified from the cell lysates. The experimental details and description of the results will be presented in a manuscript under preparation.

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

Liposomal formulation of 5-fluorocytosine using cytosine deaminase in suicide gene therapy of colorectal cancer

It is generally accepted that successful gene therapy based on suicide genes depends on two major factors: tumor-specific expression of therapeutic gene and efficient transfer of suicide genes in tumor cells. For gene-directed enzyme pro-drug therapy (GDEPT) involving

E. coli cytosine deaminase (CD) and 5-fluorocytosine (5-FC), several tumor-specific promoters and virus-based vectors were used. However, no attention has been paid to the way of 5-FC delivery to solid tumors, despite the fact that delivery of drugs to such tumors is generally low because of their insufficient transfer from the blood.

To compare the effectiveness of GDEPT with free and liposomal 5-FC, the prodrug was encapsulated in liposomes composed of dipalmitoylphosphatidylcholine (DPPC) and cholesterol. Mice treated with a dose of 5 mg of liposomal 5-FC/kg body weight for 10 days showed complete regression of transplanted tumors, whereas animals treated with the same amounts of free prodrug showed only 50% of tumor regression. When the liposomal form of 5-FC was administered i. v., complete cure was observed, but only insignificantly prolonged median survival was found when the free prodrug was inoculated by the same route.

In summary, our results showed remarkable enhancement of the antitumor effects by liposomal form of 5-FC in comparison to the free prodrug. The therapy with liposomal 5-FC represents a new approach to achieving a high local concentration of prodrug for suicidal gene therapy using *E. coli* deaminase cytosine.

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

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

TrkC is a high-affinity receptor for neurotrophin-3 (NT-3). The goal of this study was to construct genetically engineered neural cells that express TrkC under the control of a doxycycline (DOX)-inducible promoter. PC12 Tet-On is a cell line derived from rat neuroendocrine tumor with expression of the reverse tetracycline transcriptional activator rtTA. MBG18 is a neural cell line derived in our lab from brain of mouse embryos and stably transfected to express rtTA under the control of the EF1alpha promoter. The aim was to engineer the cells (MBG18 and PC12 Tet-On) to express the target gene (firefly luciferase or TrkC) at a high level in response to DOX and at a low level in the absence of DOX. We studied the expression of luciferase reporter gene regulated by the original TRE promoter (containing direct repeats of 42 bp tetO sequences) or the second-generation TRE-tight promoter (containing direct repeats of 36 bp tetO sequences), where tetO are DNA sequence elements that bind dimeric rtTA proteins. We found that the DOX-induced expression of luciferase driven by the TRE-tight promoter was much higher than the expression driven by

the TRE promoter. Finally, we demonstrate that NT-3 treatment led to the activation of signaling pathways in cells showing DOX-induced expression of the TrkC receptor.

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

Antitumor properties of diastereomeric and geometric analogs of vitamin D_3

Active and less toxic vitamin D analogs could be useful for clinical applications. We recently evaluated the antitumor effect of two new synthetic analogs of vitamin D, namely PRI-2202 (24R calcipotriol) and PRI-2205 (5,6-trans calcipotriol). Since the PRI-2202 and PRI-2205 analogs administered alone only slightly inhibited tumor growth, we applied them in combined therapy with cytostatics. Our *in vitro* results showed that the synergistic effect between the vitamin D analogs and cytostatics is more pronounced when low concentrations of the latter are used. We therefore also applied low doses of cytostatics in our *in vivo* experiments. The studies were performed in a mouse mammary cancer 4T1 and a Lewis lung cancer (LLC) model. Mice bearing tumors were s.c. injected with the vitamin D analogs and i.p. with cytostatics three times per week until the end of the experiment. Statistically significant inhibition of tumor growth by the combination therapy was observed in 4T1 mammary cancer (with cyclophosphamide) and in LLC lung cancer (with cisplatin). However, we did not observe improved therapeutic effects of the combined treatment with low doses of doxorubicin and cyclophosphamide in mice bearing LLC tumors. Moreover, the combination therapy with cisplatin led to increased toxicity of such treatment which was not dependent on the calcemic activity of the vitamin D analogs.

Combination of vitamin D analog PRI-2191, imatinib, and cytostatics: the antiproliferative effects on the HL-60 leukemia cell line

One of the main problems in the chemotherapy of patients with cancer is the administration of drugs at high doses, usually resulting in side effects. Drug administration protocols include different cytotoxic drugs used in various combinations. Consequently, it is of major interest to identify combinations of compounds that enhance their individual activities.

The purpose of the study was to analyze the influence of combinations of imatinib (inhibitor of tyrosine kinase), PRI-2191 (analog of calcitriol), and cytostatics (cisplatin, idarubicin, and docetaxel) on the inhibition of proliferation of the human leukemia cell line HL-60. These compounds exert their action by means of different mechanisms. Therefore we

carried out simultaneous treatment of HL-60 cells with these drugs. We observed that PRI-2191 strengthened the effects of imatinib and cytostatics. Imatinib combined with cytostatics had an antagonistic (combined with cisplatin or idarubicin) or additive effect (combined with docetaxel). Moreover, we observed a synergistic effect of interaction in triple combinations of the cytostatics used together with 0.1 or 0.01 μg/ml imatinib and 10 nM PRI-2191.

Many anti-cancer drugs exert their therapeutic effect by inducing apoptosis in some tumor cells. Therefore we studied the influence of combinations of the compounds on the cell cycle and apoptosis of HL-60 cells. In summary, the applied combinations acted on the cell cycle more strongly than did the compounds used individually. Moreover, the calcitriol analog PRI-2191 proved to be able to abolish the antagonistic interaction between imatinib and cytostatics.

Studies on the biological and antitumor properties of conjugates of methotrexate (MTX) with macromolecular carriers (dextran, mannan, fibrinogen, albumin)

We studied the antitumor effect of our preparations in a model of P388 mouse leukemia growing s.c. A conjugate of MTX with dextran T40 showed increased antitumor activity compared with the free MTX when the preparations were administered i.v. However, this conjugate also had increased overall toxicity in our studies. The effect of both mannan and fibrinogen-based conjugates were similar to the activity of the parental drug in this model.

We then decided to test the dextran-T40-MTX conjugate in the model with the addition of leucovorin, which is a known antidote to MTX. The addition of leucovorin protection substantially reduced toxicity in the conjugate-treated mice. Most importantly, the group treated with a combined therapy with conjugate and leucovorin demonstrated better survival in comparison with control animals and with groups treated with free MTX or conjugate monotherapy.

We also studied the biodistribution of ^{125}I-labeled mouse serum albumin, bovine fibrinogen, and their respective conjugates in mice bearing mouse mammary gland cancer 4T1. It was revealed that the amount of radio-labeled native albumin, native fibrinogen, and fibrinogen-MTX conjugate steadily decreased over time in all organs without any clear, abrupt changes. Interestingly, these preparations were selectively accumulated in the tumor tissue in the first hours after i.v. administration. In contrast, radio-labeled albumin-MTX conjugate was rapidly eliminated from the blood and was increasingly entrapped in the liver. The unfavorable radiokinetics of albumin-MTX could be explained by deterioration of the protein in the process of conjugation.

Genistein and its analogs used in combined treatment: antitumor and antimetastatic activity

In the first step of our studies we examined the antiproliferative activity of two analogs of genistein (IFG-027 and IFG-043) and two polysaccharide complexes (Xyloglucan x Genistein and Schizophillan x Genistein) against the human kidney A498 cancer cell line. The studies showed that the analogs had antiproliferative activity comparable to the genistein. SCH complex, was more active than the genistein and XYL complex, and did not show any antiproliferative activity.

We also studied the influence of genistein and its analogs and complexes on the cell cycle and apoptosis of A498 cells (the tested compounds were used in concentrations 10 μg/ml). Genistein and its IFG-027 and IFG-043 analogs caused cell cycle arrest in G2/M phase and increased apoptosis of the cells. The SCH complex also increased apoptosis and induced cell cycle arrest in the S phase and the XYL complex did not have any effect on the cell cycle distribution.

We also showed that these compounds influenced the expression of αvβ3 integrins in 10 μg/ml concentrations. Genistein and the XYL complex decreased the expression of integrins by 20%, whereas the IFG-027 analog and the SCH complex decreased it by 38%. The IFG-043 analog revealed only a low influence on the expression of the integrins (decrease by 10%).

New strategies in experimental anti-tumor immunotherapy: application of genetically modified dendritic cell-based vaccines. Optimization of schedules

According to previous studies, bone marrow-derived dendritic cells (BM-DCs) were transduced using the retroviral vector pQN carrying the murine IL-2 gene. IL-2 bioactivity was determined using the MTT colorimetric assay. Transduced cells (BM-DC/IL-2) were applied as an anti-tumor vaccine and were administered peritumorally alone or in combination with BM-DCs loaded with the tumor antigen (BM-DC/IL-2+/-BM-DC/TAg). The administration of BM-DC/IL-2+BM-DC/TAg cells resulted in slight, temporary MC38 tumor growth inhibition in C57BL/6 mice (ca. 7 days of suppression compared with controls). The host anti-tumor response was investigated after the *in vitro* restimulation of splenocytes harvested from previously treated mice. The highest percentage of cytotoxic cells was observed after the second administration of BM-DC/IL-2 cells as well as the BM-DC/IL-2+BM-DC/TAg cell combination. The highest percentage of splenocytes able to produce IFN-γ was observed after the first administration and gradually decreased after the next BM-DC administration. The induction of anti-tumor response after the application of vaccines based

on transduced MC38/0 tumor cells secreting IL-2 or IL-12 (MC38/IL-2 or MC38/IL-12) was also studied. Similar to dendritic cells, MC38/IL-2 and also MC38/IL-12 cells increased the percentage of cytotoxic splenocytes after the second administration. Studies comparing the reactivity of host immune cells after administration of the cytokine-secreting tumor cells and/or cells loaded with the tumor antigen are in progress.

Genetically modified tumor cell-based vaccines

X63-Ag8.653 (mouse myeloma) and MC38 (mouse colon cancer) tumor cells transduced with IL-2 and IL-12 genes were used as vaccines in a model of MC38 mouse colon cancer *in vivo*. Consequent to previously performed studies, the same application schedules affecting long-lasting tumor growth inhibition as well as an increase in life-span were designed. The studies realized in this year were a continuation of the former research. The multiple administration of cytokine-secreting transduced cells proved to induce the most effective anti-tumor response, especially when the protocols were supported by TAg loaded DCs. It should be underline that the degree of tumor growth inhibition was dependent on the level of cytokine produced by the genetically modified cells. Using flow cytometry, the systemic anti-tumor response induced by the vaccines was also studied (e.g. the percentage of cytotoxic spleen cells and the number of cells producing $CD8^{+}$-IFN-γ) The studies were performed in collaboration with Joanna Rossowska of the Laboratory of Cellular Interactions

Laboratory of Biomedicinal Chemistry
Head: Associate 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 at developing an effective procedure for the purification of bacterial viruses.

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

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

The leading research topic of the laboratory was investigations on the biology of bacteriophages and their use in treating bacterial infections. To this end, work was conducted on collections of phages for selected antibiotic-resistant bacterial strains, including the establishment of a collection of multi-drug-resistant bacterial strains of the genus *Stenotrophomas*. A total of six phages reactive to this bacterial strain were isolated. Moreover, an original collection of 10 phages for strains of *Enterococcus faecalis* was created and its effectiveness on 300 strains of this bacterium investigated.

In our continuing studies on the presence of phages in the human alimentary canal, it was found that the frequency of occurrence of *E. coli* phages in the intestines is lower in patients with Crohn's disease, ulcerative colitis, and intestinal cancer, although their titers might be higher.

A study on the effectiveness of phage therapy in cases of inflammation of the lactiferous gland in cows caused by *S. aureus* indicated a possibility of eliminating the staphylococci as well as of achieving a distinctly milder disease state (decline in milk leukocyte counts).

Our studies on the immunomodulatory effects of phages were continued on an autoimmunological model of collagen-induced arthritis in mice. It was found that T phage administered before and shortly after induction of the disease caused significant alleviation of its symptoms.

We had demonstrated before that phages can suppress the formation of metastases in experimental melanoma in mice. In *in vitro* studies we showed that the T4 phage mutant HAP1 and, though to a lesser degree, T4 itself, suppress the migration of melanoma cells (LPS caused stimulation of migration). In the same way it is possible that the mechanisms of the antitumoral effect of some phages are connected with their ability to suppress tumor cell migration.

In studies on the functions of phage capsid proteins, six genes encoding these proteins in T4 phage were cloned and the highly purified protein Hoc was obtained by its expression in

E. coli cells. The results of initial experiments suggest that Hoc may have adhesive properties because activated T lymphocytes and blood platelets can adhere to it.

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

Innate antiviral immunity of human leukocytes: effect of antibodies against cytokines

Two activities of innate antiviral immunity were studied: the resistance of peripheral blood leukocytes (PBLs) *ex vivo* to viral infection and the production of the cytokines TNF, IFN, IL-6, and IL-10. Additionally we studied the effect of antibodies against IFN-α, IFN-β, IFN-γ, and TNF-α on these two activities. The results of the experiments showed that complete resistance of PBLs to vesicular stomatitis virus (VSV) is accompanied by higher cytokine release. The antibodies, especially against IFN-α, expressed different effects in resistant and virus-sensitive leukocytes; in resistant leukocytes they stimulated, in sensitive they inhibited virus replication. These different effects may be attributed to changes in the cytokine profile, i.e. high TNF production by VSV-infected leukocytes and complete reduction of IL-6 production.

Acid-labile interferon-alpha during antiretroviral therapy in HIV-1 infection

The use of combination antiretroviral therapy (cART) in HIV-1 infection leads to a decrease in plasma viral load (VL) below the level of detection. Thus the complex process of immune system reconstitution can commence. We studied the concentrations of acid-labile interferon-alpha (al-IFN-α) during immune reconstitution to establish its value as a potential candidate for predicting the efficacy of cART and as a marker for ART intensification. The trial included 121 HIV-1-positive patients initiating antiretroviral therapy. Plasma HIV RNA, CD4 T-cell count, and al-IFN-α concentrations were tested every three months. The serum level of interferon (IFN) was measured using a biological assay. Our results showed that al-IFN-α concentrations correlated better with CD4 T-cell rise than did VL. The rapid and persistent decline of al-IFN-α levels better reflected the CD4 T cell count increase and preceded its rise. The study indicates the possibility of using other markers reflecting immune restoration, especially in its early phase. In some cases, based on the results of al-IFN-α concentrations, intensification of cART should be considered to inhibit immune activation due to increased CD4 T-cell turnover despite the inhibition of viral replication below the detection limit.

Activity of leukocytes from the low respiratory tract of patients with bronchial asthma

The production of nitric oxide (NO) and interferons (IFNs) were considered in studies on the activity of leukocytes from the low respiratory tract of patients with bronchial asthma. The results of the study pointed to an excessive production of nitric oxide by airway leukocytes and a damaging effect of NO on alveolar epithelial cells. This suggests that in the course of asthma, airway leukocytes, via production of NO, may contribute to epithelial impairment. Study of production of IFNs α, β, and γ by airway leukocytes of patients with asthma with particular reference to smoking habit, coexisting infection with rhinovirus, and inhaled corticosteroid treatment indicated that cigarette smoke or rhinovirus (HRV16) infection can modify IFN production in different ways depending on the subtype of IFN and corticosteroid therapy. The enhanced levels of IFN-β and IFN-γ along with a deficiency in IFN-α amount in corticosteroid-treated asthmatics who smoke or in response to HRV infection suggest that IFNs might have some relevance to pathogenic processes in asthma.

Cytokine production by human leukocytes exposed to biomaterials

Biomaterials with a high level of biocompatibility and the products of their biodegradation should not have any influence on the immunological system of an organism because such stimulation may cause an increase in inflammatory reactions as well as provoke allergic reactions. Investigation of cytokine production by human blood cells in contact with new biomaterials can be useful in identifying materials better suited for prosthesis and bone implants. This study looked into the effect of biomaterials on the production of inflammatory cytokines (IL-6, IL-8, TNF-α). Leukocytes isolated from healthy donors were exposed to biomaterials; evaluation of the local soft tissue reaction after implantation was also the aim of this study. The monitoring of the stimulation of these mediators could give us answers about the potential early and late cell reactions to the new biomaterials and could prove to be a sensitive test for the practical selection of such materials.

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

Cytokines in endometriosis

Endometriosis is a common disease among women of reproductive age (5-15% of the general population) and is characterized by the presence of endometrial-like tissue outside the uterus. It causes abdominal pain, dysmenorrhea, dyspareunia, and infertility. The

pathogenesis of endometriosis is related to functional changes in CD3+ and CD14+ cells observed both at the local and systemic level. We investigated whether the body compartment may influence cytokine expression in stimulated peritoneal and peripheral CD3+ and CD14+ cells of women with endometriosis. Isolated peripheral blood (PB) and peritoneal fluid (PF) mononuclear cells from women with endometriosis were cultured under non-adherent conditions and stimulated with PMA and ionomycin to induce intracellular cytokine synthesis of TNF-alpha, IFN-gamma, and IL-8 by CD3+ cells or with LPS to produce TNF-alpha, IL-6, IL-10, MCP-1, and IL-8 by CD14+ cells. The percentages of CD3+ cells positively stained for TNF-alpha and IFN-gamma were significantly higher and those stained for IL-8 were significantly lower in PF compared with PB, this being independent of the stage of endometriosis. In contrast, the percentages of CD14+ cells producing TNF-alpha, IL-6, IL-10, MCP-1, and IL-8 were significantly higher in the PB than in the PF of women with endometriosis. In conclusion, we were able to show that the intravascular and peritoneal compartments differentially influenced cytokine synthesis at the single-cell level in lymphocytes and monocytes/macrophages in women with endometriosis. It remains to be resolved if the observed behavior of the immune cells is characteristic of the disease per se or is rather connected with the transition of the blood–tissue barrier, independent of existing pathology.

The role of estrogen receptors in immune response

The presence of estrogen receptors in leukocytes enables the direct hormonal regulation of immune response. We showed for the first time the existence of a type I interferon pathway of modulating estrogen receptor α (ERα) expression in monocytes and macrophages. Estrogen receptor α was semiquantitatively assayed by flow cytometry using indirect intracellular staining. Macrophages isolated from mouse of the B6D2F12 strain and undifferentiated monocytes of the THP-1 cell line were cultured with different doses of INF-τ (kindly provided by Prof. F.W. Bazer) for 24 hours. In the THP-1 cells the influence of a JAK/STAT pathway inhibitor (AG-490) was assessed. In these cells, IFN-τ induced STAT2 nuclear translocation, which was confirmed microscopically. ERα was labeled in harvested cells and analyzed after acquisition by comparison of specific MFI. We showed that 100 ng/ml of IFN-τ increased the ERα-specific fluorescence in THP-1 cells compared with unstimulated cells. This reaction was reversed by addition of AG-490. On the other hand, in mouse macrophages we observed downregulation of ERα level compared with unstimulated cells. Our observations were confirmed in the mouse J744E macrophage cell line with increasing doses

of IFN-τ. We did not observed a linear dose-dependent influence of IFN-τ on ERα receptor level. Our results provide evidence that IFN-τ can regulate ERα level in monocytes and macrophages. This effect is dependent on activation of the JAK/STAT signaling pathway.

Laboratory of Cellular Interactions
Head: Associate Professor Danuta Duś, Ph.D.
Cancer cell-endothelial cell interactions during tumor progression

The variable outcome of cancer patients with similar clinical status creates a need for the search for new and reliable prognostic indicators of tumor progression, recurrence, and survival.

During blood-borne metastatic spread, extravasation of tumor cells is a prerequisite for distant tissue colonization. Necessary for extravasation are close adhesive interactions between the cancer cell with endothelial cells at the site of cancer cell extravasation. Our studies are aimed at endothelial cells: their phenotypic characteristics and, particularly, their activation mechanisms and organ-specific adhesive interactions under normal and pathological conditions.

Endothelial cells are critical in the recruitment and migration of circulating effector cells into sites of inflammation and necrosis. Endothelial cell injury occurring in a variety of pathologies could result from the cytotoxicity of effector lymphocytes. We showed that human NK cells were able to adhere and kill human endothelial cell (EC) cell lines. This organ-specific NK-mediated endothelial cell killing occurs in an IL-2 activation-dependent manner. The study on the cytotoxic pathway used by NK cells indicated a predominant role for the perforin/granzyme pathway in endothelial cell death. The human NK cell cytotoxicity toward ECs may be a potential target to block vascular injury or, alternatively, to destroy the pathological vessels involved in the development of invasive pathologies such as cancer.

Our current studies on endothelial cells (ECs) are aimed at their phenotypic characteristics, particularly their activation mechanisms and organ-specific adhesive interactions under normal and pathological conditions. Stem cells in umbilical cord blood present a greater proliferative capacity than cells derived from adult blood or bone marrow. Therefore, the aims of our study were: 1) to elaborate the culture conditions for recurrent *ex vivo* expansion of EPCs isolated from human umbilical cord blood and 2) to prove that under the conditions used, the EPCs growing *in vitro* remain capable of differentiating into phenotypically mature, functional endothelial cells. CD133-positive cells were isolated from umbilical cord blood by immunomagnetic sorting and further expanded and differentiated *in*

vitro for up to 4 weeks in the presence of cytokines and growth factors. After 10 days of culturing the CD133-positive cells, an approximately 30-fold expansion of cell number in culture was observed, and on day 22 of culture an approximately 80-fold expansion of total nucleated cells number was achieved. The morphological features of the cultured cells were evaluated. The phenotypic characteristics of the cultured cells were assessed by flow cytometry, immunocytochemistry, and reverse transcription polymerase chain reaction (RT-PCR).

Freshly isolated CD133-positive cells express CD34 CD105, CD117, and CD31 endothelial precursor cell antigens and KDR (VEGFR-2) as well as the multidrug resistance-conferring proteins MDR1, MRP1, MRP3, and BCRP. *In vitro* cultured CD133-positive cells acquired an endothelial phenotype which was marked by the appearance of VE-cadherin and von Willebrand factor expression and decrease of CD133, CD34, CD105, CD31, MDR1, and KDR expression. Differentiated cells were able to form pseudovessels in reconstituted extracellular matrix. These studies provide strong evidence for the existence of different subpopulations of endothelial cell precursors in cord blood and suggest the use of *ex vivo*-expanded cord blood endothelial precursor cells as a tool for the investigation of postnatal lineage diversification.

Studies on endothelial cells were performed in collaboration with Dr. Claudine Kieda, CBM CNRS UPR 4301, Orleans, France, and Dr. Salem Chouaib, Institut Gustave Roussy, INSERM U487, Villejuif, France.

Laboratory of Tissue Immunology
Acting head: Assistant Professor Beata Nowakowska, Ph.D.
The rare allele HLA locus B in the south-west Polish population

Microchimerism in kidney transplantation

The presence of donor-derived hematopoietic cells in the blood and various tissues of organ recipients, termed allogeneic microchimerism, has been considered to play an essential role in establishing organ acceptance. However, it remains unclear whether this peripheral donor-cell microchimerism plays an active role in graft acceptance or is simply a consequence of maintaining sufficient immunosuppression to avoid rejection. In our study, microchimerism was detected in 12 of 32 female recipients of male kidneys. There was no significant correlation between the presence of peripheral blood microchimerism and age at transplantation, renal function, or episodes of acute rejection and HLA-DR matching. The

results demonstrate that this is a dynamic process and may not be detectable at one point in time.

Association of HLA DRB, DQA1, and DQB1 alleles with response to interferon-alpha therapy in children with chronic hepatitis B

HBV-infected children are at high risk of chronic hepatitis. Genes of the HLA system are among the important genetic factors involved in the pathogenesis of this infection. The aim of the study was to investigate the association between HLA alleles and the results of interferon-alpha therapy. We found that the group with sustained complete response to therapy was characterized by significantly lower DRB5* allele frequencies than the children with partial or no response (20% vs. 35%, p=0.0441). Patients with HBeAg elimination but with HBV-DNA-positive DRB1*01 alleles had significantly lower frequencies than the group without any improvement after therapy (11% vs. 28%, p=0.0146). HLA DQA1* 0501 carriers showed predisposition to HBeAg clearance (p=0.0222), but not to HBV-elimination. The HLA DQB1*0502 allele and DQB1*0502 – DRB1*16 haplotype were associated with unresponsiveness to interferon-alpha therapy (p=0.0213 and p=0.0180, respectively).

Genetic predisposition to cancer development

Our studies concerned the molecular basis of familial cancer aggregations of a complex phenotype known as Li-Fraumeni syndrome (LFS) and Li-Fraumeni-like syndrome (LFL). The spectrum of cancers in these families comprises mostly breast cancers, bone and soft tissue sarcomas, brain tumors, and leukemias. In such families, the high risk of cancer is associated with germline p53 mutations. In our set of LFS/LFL families, germline p53 mutations were very rare. Therefore, our recent studies concerned germline CHK2 mutations. They were also found at low frequency. Moreover, due to the occurrence of CHK2 mutations in healthy control persons, their involvement in the pathogenesis of LFS/LFL is uncertain.

Genetic polymorphisms and ovarian cancer

DNA sequence variation may explain both predisposition to cancer development and some of the interindividual differences in drug-induced adverse reactions and therapeutic responses. Among the genes which may have an impact on patient response to chemotherapy are those coding for drug-metabolizing enzymes, ABC transporters affecting both drug biodistribution and cancer sensitivity to chemotherapy, and proteins involved in DNA repair. We investigated single-nucleotide polymorphisms (SNPs) in several genes of the above

classes in relation to ovarian cancer. In general, the frequencies of the studied alleles and genotypes were similar in cancer patients and a control group. In the case of the NAT2 gene, genotypes coding for rapid acetylation were less frequent among cancer patients than a control group (32.6 vs. 48.2%). In contrast, genotypes coding for slow acetylation were more frequent among patients than controls (67.4 vs. 51.8%). These differences were statistically significant (*RR*=1.4, 95%*CI*=1.08-1.8, *p*=0.01). Detailed analysis showed that in the patient group the two genotypes *5/6 (*RR*=1.53, 95%*CI*=1.16-2.02, *p*=0.004) and *6/6 (*RR*=1,63, 95%*CI*=1.15-2.3, *p*=0.04) were frequent. The data pointed to an association between slow acetylation and ovarian cancer development. Analysis of the data in respect to chemotherapy toxicity and efficacy showed no significant correlation.

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

Laboratory of Transgenesis and Lymphocyte Biology
Head: Professor Paweł Kisielow, Ph.D.
Epigenetic regulation of RAG-2 intragenic promoter of *NWC*

NWC, a new gene discovered in our laboratory, represents the third evolutionarily conserved gene within the *RAG* locus. *NWC* is transcribed at a high level in all cells except T and B lymphocytes and their *RAG*-negative progenitors. This is because the expression of *NWC* in lymphocytes is regulated by the *RAG-1* promoter, while in all other cells it is controlled by its own *RAG-2* intragenic promoter, which in T and B lymphocytes is silent. Our recent experiments were focused on elucidating the mechanisms responsible for the lymphocyte-specific inactivation of the *RAG-2* intragenic promoter of *NWC*. We found that its inactivation in lymphocytes is caused by CpG island hypermethylation, which is accompanied by site-specific blocking of chromatin accessibility. However, the change in chromatin accessibility at the site of the *NWC* promoter, in contrast to the *RAG-1* and *RAG-2* promoters, is not accompanied by the posttranslational modifications of histone H3 that are usually associated with transitions between transcription permissive and transcription repressive chromatin configurations. These results indicate that the accessibility of the *NWC* and *RAG* promoters to trans-acting factors is regulated by different epigenetic mechanisms. Paradoxically, the permanent repression of *RAG* genes in non-lymphocytes, in contrast to the lymphocyte-specific developmental stage-dependent downregulation of *RAG* expression, does

not involve silencing of transcriptional activity within the *RAG/NWC* locus, raising the possibility of an active role of *NWC* transcription in preventing the expression of *RAG* genes.

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

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

Laboratory of Clinical Immunology continue collaborative work with the Lower Silesian Centre for Cellular Transplantation with the National Polish Marrow Donor Registry in the field of cellular transplantation and immunogenetics.

In February 2006 a conference was organized on Immunogenetics in Haematology and Stem Cell Transplantation, in which participated scientists leading in the field of immunogenetics and haematopoietic stem cell transplantation.

The participants include: M. Petrek (Czech Republic), E. Holler, M. Uhrberg (Germany), M.A. Cook, A. Dickinson, M. Lowdell, A. Madrigal, D. Middleton, B. Shaw (Great Britain), D. Montagna, L. Ruggeri, A. Velardi (Italy), M. Griffioen**,** E. Spierings (Netherlands), K. Bogunia-Kubik, P. Kisielow, A. Lange (Poland), and the proceeding will be published in 7/8 issue of the *International Journal of Immunogenetics*.

The main assets of 2006 work include:

1) The discovery of an association between low IFNγ production genotype and the risk of CMV reactivation. This work highlighted the role of immunogenetics factors in viral reactivation which was further substantiated by another novel finding on deletion of CCR5 gene which appeared to make individuals less susceptible to aGvHD and EBV reactivation.
2) Clinically important work was devoted to elucidation of the impact of HLA matching on the outcome of transplantation. The significance of allele matching was documented and the clinical outcome of transplantation with respect to that was critically evaluated and described.
3) In contrast to previous publications from other labs, we documented that an increase of CD4+CD25+ in blood of HSCT patients follows activation of the immune system (an immense of CD134+ cells). CD4CD25 cells expanded to control alloreactivity also in a non-specific manner makes patients more susceptible to viral reactivation.

The latter was shown on a clinical ground and by enumeration of CD8 viral specific cells with the use of a novel technology (pentamer staining). Understanding of the role of Treg cells was greatly facilitated by viral studies.
Measurements of viral copies helped not only to identify patients at risk but also to document mutual interaction between immune system and viral reactivation which affects the outcome of transplantation.

The progress in our research was spread in publications and contribution to international conferences including EBMT, EFI and ASH meetings.

From practical point of view, continuation of our work on the immune system reconstitution post transplant helped also to understand the risk of relapse in chronic myeloid leukaemia.

Laboratory of Immunogenetics
Head: Professor Piotr Kuśnierczyk, Ph.D.
CTLA-4 gene polymorphism in allergic asthma

Distribution of KIR genes in a healthy Polish population and in disease

Activatory and inhibitory killer cell immunoglobulin-like receptors (KIRs) are cell surface glycoprotein molecules present on natural killer (NK) cells and subsets of T lymphocytes. They are encoded by *KIR* genes located on chromosome 19q13.4 in humans. *KIR* genes are highly polymorphic, i.e. they possess 4 to 20 alleles each. *KIR* genes are characterized by a haplotypic polymorphism, i.e. individual chromosomes differ with respect to the number and nature (activating versus inhibitory) of *KIR* genes. Therefore, individuals bearing different repertoires of *KIR* genes may differ in their susceptibility to infections, neoplasms, and autoimmune diseases. In addition, the *KIR* gene repertoire influences the outcome of hematopoietic stem cell transplantation. Therefore it is important to know the distribution of *KIR* genes in a given human population. We established the frequencies of *KIR* genes in about 500 healthy, unrelated, Polish Caucasians and found them to be similar to neighboring European populations. We recalculated our earlier data on *KIR* gene associations with psoriasis vulgaris and found interesting protective effects of several *KIR* genes. We also found interesting associations of *KIR* genes with distinct clinical manifestations of rheumatoid arthritis, suggesting different genetic backgrounds of particular forms of the disease. Participating in a project run by the Department of Hematology and BMT, Silesian Medical University, Katowice, we found that an increased number of activating *KIR*s in the donor, in particular in the absence of their counterparts in the recipient, seems to enhance GVH

alloreactivity, resulting in impaired survival. Finally, we obtained a result suggesting a protective effect of the *KIR2DS5* gene in endometriosis.

Associations of the PTPN22 single-nucleotide polymorphism 1858C>T with rheumatoid arthritis and in allergic asthma

The protein tyrosine phosphatase nonreceptor type 22 (*PTPN22*) gene encodes an intracellular lymphoid tyrosine phosphatase involved in the inhibition of T-lymphocyte activation. Its single-nucleotide polymorphism (SNP) 1858C>T was found to be associated with multiple autoimmune diseases, particularly those with a contribution of autoantibodies. Therefore, *PTPN22* is considered a gene of autoimmunity. We examined the distribution of 1858C>T alleles in rheumatoid arthritis, in allergic asthma, and in healthy control individuals from a Polish Caucasian population. We found associations between several clinical manifestations of RA and the *PTPN22* 1858T allele. However, no association with the 1858C>T polymorphism was found for susceptibility to allergic asthma or for severity of the disease. This is the first study of the *PTPN22* polymorphism in allergic asthma.

TGFB1 polymorphisms in allergic asthma

Allergic asthma is a complex genetic disorder that involves interactions between genetic and environmental factors. Some studies have indicated that TGF-β1 may participate in the pathogenesis of asthma. Several polymorphisms have already been described in the *TGFB1* gene; some were tested in allergic asthma, with conflicting results. The aim of this study was to investigate the possible associations of four *TGFB1* gene polymorphisms with allergic asthma in a Polish population by means of the PCR-RFLP method. Significant results for three of the studied SNPs were obtained. These results suggest a contribution of the *TGFB1* gene to the allergic asthma phenotype and to severity of the disease in the Polish population.

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

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

Multiple myeloma (MM) is a B-lineage malignancy characterized by an accumulation of isotype-switched immunoglobulin-producing monoclonal plasma cells. Phenotypic and functional abnormalities of T cell are also observed. The implication of T cells in the

development of plasmocytomas has been shown in a murine model. The association between polymorphisms of the genes encoding major T-cell costimulatory (CD28, ICOS) and downregulatory (CTLA-4) molecules and susceptibility to MM in a Polish population was studied. One hundred patients with MM and 202 healthy subjects were studied. Genomic DNA was isolated from whole frozen blood using the NucleoSpin^{R} Blood kit. Allele identification was achieved by PCR amplification. The amplified product for SNP loci was purified and minisequenced using the commercial kit SNapShot (PE Applied Biosystems). The dinucleotide repeat polymorphism was studied by PCR and a fluorescence-based technique. The products were analyzed on an ABI PRISM 310 Genetic Analyzer (ABI PRISM 310 capillary electrophoresis system). An association of the *CTLA-4* 49G and $(AT)_{82}$ alleles in 3'untranslated region with MM (p=0.018, *OR*=1.57, 95%*CI*=1.08-2.29 and p=0.0013, *OR*=1.85, 95%*CI*=1.27-2.72, respectively) was found, while no association between T/C substitution in intron 3 of the *CD28* gene, the dinucleotide (GT)n repeat polymorphism in intron 4 in *ICOS* gene, and C/T base exchange in the promoter (position -318) of the *CTLA-4* gene and MM was observed. The results indicate that the A/G substitution in exon 1 and the microsatellite polymorphism in exon 4 in the 3'untranslated region of the *CTLA-4* gene might be a susceptibility locus for MM. This study is the largest and most comprehensive to date to evaluate the association between genetic polymorphisms of genes encoding T-cell costimulatory and downregulatory molecules and susceptibility to MM.

Graves' disease (GD) is an autoimmune disease caused by environmental and genetic factors. The *CTLA-4* gene encoding the negative regulator of the T-lymphocyte immune response is a candidate gene for conferring susceptibility to thyroid autoimmunity. The association between the recently described gene polymorphisms *CTLA-4*/CT-60(G/A) and *CTLA-4*/JO-31(G/T) in the 3' UTR conservative region and susceptibility to GD and Graves' ophthalmopathy (GO) in a Polish population was estimated. Altogether, 98 unrelated Polish patients with GD (49 with clinically evident GO, NOSPECS class $\geq$3) and 135 healthy subjects were genotyped. Allele identification was achieved by PCR amplification followed by minisequencing with the commercial kit SNapShot (PE Applied Biosystems). The products were analyzed by capillary electrophoresis on an ABI PRISM 310 Genetic Analyzer. The distribution of the allele, phenotype, and genotype frequencies of *CTLA-4*/CT-60 and *CTLA-4*/JO-31 did not differ between patients with GD and healthy subjects or between GD patients without, with GO, and controls. However, there was a significantly higher frequency

of the G-allele (*CTLA-4*/JO-31) in patients with severe GO compared with patients without GO (65% vs. 51%, Fisher's exact test p=0.05) and the G-positive phenotype (89% vs. 69%, Fisher's exact test p=0.01). Haplotype analysis showed that patients carrying a G haplotype were more prone to GD (p=0.04, *OR*=2.38) and the appearance of ophthalmopathy (p=0.01, *OR*=3.15) than individuals carrying others haplotypes. These results suggest that the *CTLA-4* gene might play a role in the susceptibility to Graves' disease and risk of GO.

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

Studies were conducted on the VPRSGEVYT fragment located in the exposed loop of the HLA-DR molecule. New dimeric analogs were constructed (Prof. Szewczuk, University of Wrocław, Poland) where VPRSGEVYT peptides were linked by their N-termini by means of polyethylene linkers of various length to mimic the immunosuppressive fragments of class II HLA molecules. The results showed that the dimerization of the peptides led to increased immunosuppressive activity in the model of secondary humoral immune response of mice *in vitro* and the activity of the peptides depended on the linker length.

Investigations were also continued aimed at demonstrating the immunotropic activities of isoxazole derivatives, synthesized by Prof. Ryng (Medical University, Wrocław, Poland). The compounds were tested in several experimental models, such as secondary immune response *in vitro* to SRBC, proliferative response of splenocytes to concanavalin A, and lipopolysaccharide-induced cytokine (TNF-alpha and IL-6) production. The studied compounds exhibited individual activity profiles in the described tests (mainly immunosuppressive). The four investigated compounds represent lead structures which will be further modified to obtain compounds of potential therapeutic value.

In other studies we investigated the immunological activity of a cyclolinopeptide (CLA) and its modifications synthesized by Prof. Zabrocki (Technical University, Łódź, Poland). The modifications of the CLA molecule may lead to acquiring new interesting properties of therapeutic potential. It appeared that the effects of CLA on the humoral immune response in mice were strictly dose dependent, i.e. low doses stimulated and high doses inhibited the response. However, when CLA was used together with methotrexate (MTX), an additive, suppressive effect was observed even at low doses of CLA. Such a result may be of importance since it creates the possibility of achieving immune suppression even at low

(nontoxic) MTX doses. The basic structure of CLA was devoid of antagonistic (probably anti-apoptotic) activities against MTX. Thus the immunotropic characteristics of CLA resembled those of a classical immune suppressor, cyclosporine A. In addition, application of CLA derivatives with MTX showed that these compounds had antagonistic properties with respect to MTX.

Studies on the efficacy of bacteriophage treatment in mice infected with *Escherichia coli* and *Staphylococcus aureus* showed equal protective action of specific bacteriophages administered intraperitoneally or *per os*. In addition, we showed that intravenous pretreatment of mice with lactoferrin, an iron-binding protein from milk, allowed applying very low bacterophage doses (10^3-10^4/mouse) in order to achieve a similar reduction in the number of bacteria in the liver as to application of a high (10^8) number of bacteriophages.

The role of different factors affecting the skeletal system

For the last six years the main topic of our investigations have been bone remodeling processes. The study of the effect of osteopontin on the activity of osteoclast-like cells derived from a blood mononuclear cell population proved their ability to digest bone digestion. This feature was useful in investigating the activity of osteoclast precursor cells derived from the blood of patients with menopausal osteoporosis evoked by long-lasting treatment with steroids or of unknown etiology. Significant differences in the osteolytic activity of osteoclast-like cells were discovered. The use of heterotopically induced osteogenesis in the investigations of osteoporosis as well as in the treatment of local skeletal defects showed the major role of seven isoforms of bone morphogenetic proteins in this process. Evaluation of the activities of genes for bone morphogenetic proteins in the axial and peripheral skeleton in bone regeneration lead to a better understanding of bone healing. Osteogenesis, induced by autogenic cells transfected by genes of three bone morphogenetic protein isoforms, BMP-2, -4, and -6, could be useful in the clinical treatment of bone defects caused by bone diseases or fractures. Fibroblasts were chosen for this model as the most suitable cells.

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

Laboratory of Medical Microbiology
Head: Professor Andrzej Gamian, Ph.D.
Studies on the pathogenesis of some diseases of bacterial etiology and a role of bacterial surface glycoconjugates and protein antigens in immune response

The current activity of the laboratory is focused on studies of the mechanisms of the pathogenicity of diseases with bacterial etiology, the role of molecular mimicry, bacterial proteins and glycolipids in pathogenicity, and the structures and functions of bacterial capsular antigens and endotoxins. In the search for molecular markers of bacterial infections and prognostic factors, a method for the determination of endotoxins has been developed which relies on determining the chemical marker of endotoxin, the 2-keto-3-deoxy-octulosonic acid (Kdo), using GLC-MS. Another approach for endotoxin detection concerns biosensor applications of luminescence depolarization effects. The monitoring of specific markers for sepsis and septic shock could significantly facilitate the prognosis of these diseases and their treatment. Consequently, a third approach relies on the detection of another marker of endotoxin, 3-hydroxy fatty acids, which allows measuring its level in patients' sera. The general strategy for the development of protective tools against invading bacteria comprises determining the structures of the molecules involved in an infection and the immune processes, their chemical and genetic manipulations, as well as understanding their biological activities. The structures of such antigens have been established as well as a new method for isolating glycolipids using supercritical carbon dioxide. Studies on the protective properties of enterobacterial outer membrane proteins revealed a single compound responsible for this activity, namely a 38 kDa outer membrane protein common in *Enterobacteriaceae* strains. This bacterial protein was found to be a useful diagnostic marker of specific immunodeficiencies, but may also be considered as a carrier for safe and effective vaccines.

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