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Research Report 2007

Laboratory of Bacteriophages
Head: Professor Andrzej Górski, M.D.
Research on the biology of bacteriophages and their use in the treatment of bacterial infections

Research into the isolation of phages specific to *Stenotrophomonas maltophilia* strains was continued utilizing 42 environmental samples. Seven specific bacteriophages which did not demonstrate lytic activity to other microorganisms of the *Pseudomonadaceae* family were isolated. Electonmicroscopic examination of the phages showed that they belonged to two morphological types of the *Myoviridae* family: one to type A1 and six to type B1.

A purified preparation of T4 phage showed a dose-dependent inhibitory effect on free-radical production by polymorphonuclear leukocytes stimulated by the LPS of *E. coli*.

There was no visible anti-metastatic activity of purified Hoc protein from T4 phage in mice treated with the protein one hour before B16 melanoma cell graft and no effect of protein gp Hoc on the migration of these cells.

A retrospective analysis was conducted of certain parameters of inflammation, i.e. sedimentation rate, CRP, and leukocytosis, in patients with chronic, symptomatic, antibiotic therapy-resistant bacterial infections who qualified for phage treatment within the project "Experimental Phage Therapy of Antibiotic Therapy-Resistant Infections, Including MRSA Infections". Data from 30 patients with inflammation of the bone, peri-prosthesis, or soft tissue, in the majority of cases induced by *S. aureus*, were analyzed. No significant changes in mean serum levels of CRP were observed in the first week of phage therapy compared with baseline CRP levels measured before therapy began (32.6 vs. 27.9 mg/l, n=11). However, significant ($p<0.05$) decreases in CRP level measured between the 7^{th} and 30^{th} days of phage therapy (27.1 vs. 19.8 mg/l, n=30) were noted. After completion of therapy, the mean serum CRP level was lower as well (25.7 vs. 18.6 mg/l, n=17), although this result was not statistically significant. Similar tendencies were observed in changes in mean leukocytosis values, but mean ESR in the patients before, during, and after therapy did not change significantly.

Review of results of grant activities

Significant antibacterial, protective, and immune modulatory activities of phages were demonstrated in mice after oral administration.

A collection of bacteriophages specific to *Enterococcus faecalis* was established.

Antitumoral activity of a selected mutant T4 phage for *Escherichia coli* (HAP1) was demonstrated in experimental mouse models as was a synergistic antitumoral effect of T4 phage in conjunction with cyclophosphamide and a vitamin D analogue.

A significant effect of phages was shown on increasing survival of experimental skin allografts in mice as well as a decreased activation of NF-κB transcription factor, which suggests the possibility of employing phages in transplantology. The preliminary results indicate that bacteriophages may interfere with the cytopathogenic effect exerted by adenoviruses and inhibit the activation of NF-κB induced by them.

It was shown that administering the *E. coli* bacteriophage T4 to mice DBA-1 may decrease the intensity of articular symptoms in a model of rheumatic inflammation of the joints. Purified preparations of phages for Gram-positive and Gram-negative bacteria acted in an immunosuppressive manner both *in vitro* and *in vivo* (e.g. T- and B-lymphocyte activation and proliferation, cytokine and antibody production). In addition it was shown that phages may decrease free-radical production by phagocytes, which in turn suggests the possibility of their application in sepsis.

Optimization of the conditions of migration for cells of the B16 mouse melanoma cell line, the HL60 human leukemia cell line, and human blood mononuclear cells was carried out. Migration was studied with a membrane method using the matrigel technique, membranes flattened by fibronectin, and the agarose plate method. *E. coli* LPS preparations were used as controls.

Initial results of a study comparing the effects of T2 and T4 bacteriophage preparations on human blood mononuclear cell migration indicate the possibility of inhibiting migration with the T2 bacteriophage preparation, while the T4 bacteriophage preparation does not seem to affect migration.

The penetrability of orally administered staphylococcal phage (A5/80) to the blood of rats was demonstrated after prior neutralization of gastric juices with aluminum carbonate applied orally several minutes before phage administration (a similar procedure is used in phage therapy). Research was begun on optimization of phage absorption from the alimentary canal. Initial results indicate that administering mixtures of phages in the form of phage lysate and

bicarbonate increases the phages' ability to penetrate to the blood after their administration to the stomach.

Work was continued on developing optimal methods for culturing olfactory glioma cells (OGCs) for transplantation. The effect of the cell culture supernatant (so-called conditioned medium) was studied. Quicker adhesion of a larger number of the cells to plastic was observed in comparison with medium supplemented with serum or with serum and nerve growth factor, neurotrophine-3, or pituitary extract.

Increased total quantities of cultured OECs were observed in conditioned medium as were morphological changes in these cells. Adding conditioned medium from culture of OGCs isolated from the olfactory bulb caused a decrease in the number of fibroblast-like cells as well as in increase in multipolar net-forming OGCs. It was established that using conditioned medium brings a better effect (OEC culture efficiency) when it is added to the full culture medium at the beginning of cell culturing.

Research methods developed and patents applied for:

Patent applications

1. Weber-Dąbrowska B., Górski A.: Seventeen patent applications to the Polish Patent Office concerning newly isolated *Enterococcus faecalis* bacteriophages (application nos. P382771 to P382799, date of application: June 28, 2007)
2. Boratyński J., Szermer-Olearnik B., Weber-Dąbrowska B., Górski A.: Patent application to the Polish Patent Office entitled "A method of obtaining bacteriophage preparations containing trace amounts of endotoxin" (application no. P382800, date of application: June 29, 2007)
3. Siewiński M., Czecior E., Szymaniec S., Fortuna W., Międzybrodzki R., Tarnawa R., Pieniek A.: Patent application to the Polish Patent Office entitled "The application of cysteine peptidase inhibitors to mark free cells or those which are part of tumor tissue". (application no. P381888, date of application: March 2, 2007)

Awards, distinctions:

1. Prof. Andrzej Górski: The ”*National Geographics*” award for progress in phage therapy.
2. Dr Krystyna Dąbrowska: ”*Program Start*” of the Foundation for Polish Science for the annual scholarship for young scientists in 2007.
3. Dr Wojciech Fortuna and Dr Ryszard Międzybrodzki: Team Award of the Ministry of Health for Academic Teachers in 2007 for the publication: ”*The olfactory bulb and olfactory mucosa obtained from human cadaver donors as a source of olfactory ensheathing cells*”.

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

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

Towards an understanding of the function of* NWC*, the third evolutionarily conserved gene within the RAG locus, and identification of its protein product

We previously showed that the transcription of *NWC* is differently regulated in lymphocytes than in non-lymphocytes and characterized in detail the *NWC* promoter region as well as the mechanism regulating its activity. It was found that in lymphocytes, the *NWC* promoter is silenced by methylation of the CpG island present in that region and that the regulation of *NWC* transcription is overtaken by the *RAG1* promoter. Based on this knowledge we have begun to prepare several genetic constructs to generate transgenic mice in which the influence of *NWC* transcription on the expression of *RAG* genes in lymphocytes and in non-lymphocytes will be studied. Using bacterial artificial chromosomes (BACs) containing the entire *RAG/NWC* locus in which the *RAG2* gene is fused with the green fluorescent protein (GFP) gene and *NWC* gene is fused with the yellow fluorescent protein (YFP) gene by ET recombination. The analysis of transgenic mice obtained with this construct (BAC-NY) should allow simultaneous monitoring of the activity of the *RAG* and *NWC* genes in different cells during normal development. In addition to the potential of providing new information concerning the developmental pattern of gene expression within the *RAG/NWC* locus, BAC-NY transgenic mice will serve as a 'normal' control for transgenic mice obtained with BAC constructs containing genetically manipulated *NWC* in which the *NWC* promoter is deleted (BAC-NY-ΔpNWC) or replaced by a promoter constitutively active in all cells, including lymphocytes (BAC-NY-pEF1alpha). These last two BAC constructs made by manipulating the BAC-NY construct are in the final stage of preparation.

Rat monoclonal antibody raised against recombinant NWC protein identifies an acidic 37-kDa protein in mouse tissue extracts. Preliminary results indicate that the protein shows both nuclear and cytoplasmic localization.

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

Hafnia alvei and its LPS (lipopolysaccharide, endotoxin) are among the reported causative agents of bacteremia and septicemia in humans and animals. These Gram-negative, peritrichously flagellated rods are oxidase negative, nonsporulating, produce nitrate reductase, and are positive for enterobacterial common antigen. Nineteen to 42 cases of bacteremia per year were reported in the United Kingdom between 2001 and 2003. Most of them were monomicrobic infections and in approximately 33% of the cases *H. alvei* was isolated not only from the blood, but also from hepatic abscesses, pancreatic pseudocyst fluid, sputum, feces, and central venous catheter. Besides bacteremia and sepsis, *Hafniae* are also associated with respiratory diseases and mixed hospital infections in humans. *H. alvei* was the third most common enteric species identified, after *Escherichia coli* and *E. cloacae.* Since the gastrointestinal and respiratory tracts represent very common habitats for *Hafniae*, most cases of bacteremia caused by *H. alvei* usually originate there. Sepsis seems to be the most common syndrome reported in the case of *H. alvei* infection, and LPS represents its main virulence factor. Most of the elucidated structures of *H. alvei* LPS are smooth-type molecules built up of O-specific polysaccharide (PS), core oligosaccharide (OS), and lipid A, the center of the biological activity of the endotoxin recognized by cells of the immune system.

The O-antigens of *H. alvei* are subdivided into 40 O-serotypes. Recently, the structures of the O-specific polysaccharides from 30 serologically different *H. alvei* strains have been elucidated, showing some unusual constituents such as aminodideoxyhexoses (Qui4NAc, Qui3NFo), phosphodiester groups, glycerol-3-phosphate groups, phosphoethanolamine, and sialic acids.

So far four types of core OS were identified in *H. alvei* LPSs. The most common core oligosaccharide isolated by mild acidic hydrolyses from the LPSs of smooth *H. alvei* strains is a hexasaccharide composed of two D-glucoses, three L,D-heptoses, and one 3-deoxy-octulosonic acid molecule (Kdo). Two heptose residues are substituted by phosphoethanolamine and phosphoryl groups.

Kdo-containing fragments other than the known structures of core oligosaccharides were previously found among fractions obtained by mild acid hydrolysis of lipopolysaccharides isolated from some strains of *H.* alvei, but the position of such fragments in the LPS structure was not known to date. Analyses of de-*N,O*-acylated LPSs with the use of NMR spectroscopy and mass spectrometry allowed the determination, for the first time, the location of Kdo-containing trisaccharide in structures of *H. alvei* 32 and 1192 LPSs. Trisaccharide [L-α-D-Hepp-(1→4)-[α-D-Galp6OAc-(1→7)]-α-Kdo-(2→] has been found to be an integral, but acid labile part of the outer core oligosaccharides of these LPSs. The screening for Kdo-containing trisaccharides was performed on the group of 37 O-serotypes of *H. alvei* LPSs using monospecific antibodies recognizing this structure. The trisaccharide is a characteristic feature of the outer core oligosaccharide of *H. alvei* 2, 32, 600, 1192, 1206, and 1211 LPSs, but six weaker cross-reactions suggest the presence of similar structures also in LPSs of the strains 974, 1188, 1198, 1204, and 1214. Thus we have defined a new example of enterobaterial endotoxins among those elucidated so far. This type of core oligosaccharide deviates from the classical scheme by the presence of its structural motif with Kdo in the outer core. Lipid A isolated from *H. alvei* endotoxins was analyzed for the first time with the use of ESI MS^n and MALDI-TOF mass spectrometry. Both lipids A contained glucosamine backbone phosphorylated at the 1 and 4' positions. The disaccharide backbone was acylated by 14:0(3-OH) at positions 2 and 3. Positions 2' and 3' are substituted by 14:0(3-O-12:0) and 14:0(3-O-14:0), respectively.

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

Several lines of evidence indicate that sialyl $Lewis^a$ and sialyl $Lewis^x$, tumor-associated carbohydrate antigens present on several types of human tumors, are involved in the formation of metastases. In our previous studies we showed that sialyl $Lewis^a$ is important in the development of liver metastases by colon cancer cells. To study the role of the sialylated Lewis structures in the development of organ-specific metastases by breast cancer cells, in the present work we have used two cell lines with well-defined tropism to specific organs. MCF10-IV cells metastasize to the lung and the BO2 derivative of MDA-MB 231 cells only to bone. To create a specific "gain-of-function" phenotype, BO2 cells, which lack sialylated Lewis structures, were transfected with α1,3/4-fucosyltransferase using a lentivirus system.

The resulting population of BO2 cells expressing sialyl Lewis^a and sialyl Lewis^x were studied for the formation of metastases after their intracardiac implantation into athymic nu/nu mice. It was found that sialyl Lewis^a/sialyl Lewis^x-positive breast carcinoma BO2 cells, in contrast to the parental cells, develop metastases not only in bones, but also in the lung and mesenteric lymph nodes. To create a "loss-of-function" phenotype, MCF10-IV cell were used. These cells were transfected with shRNA using a lentivirus system to inhibit the expression of Fut3 in sialyl Le^a/sialyl Le^x-positive cells. After intracardiac injection of MCF10-IV cells with suppressed expression of sialylated Lewis structures, nu/nu mice developed metastases not only in the lung, but also in mesenteric lymph nodes. Therefore our data further support the thesis of the importance of sialylated Lewis structures not only in the development of metastastases, but also in their organ-specific localization.

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

Proline-rich polypeptide complex (PRP) isolated from ovine colostrum shows immunoregulatory and procognitive activities. In the form of orally administered tablets (Colostrinin containing 100 μg of PRP) it improves the outcome of Alzheimer's disease (AD) patients. The mechanism of action of PRP/Colostrinin in AD is not yet fully clarified. It is known that neurodegenerative processes may be enhanced by oxidative stress and overproduction of NO and proinflammatory cytokines. It was previously shown that PRP regulates the secretion of Th1 (IFN, TNF-α) and Th2 (IL-6, IL-10) cytokines, inhibits the release of nitric oxide, and affects iNOS activity.

Another product of cell metabolism, hydrogen peroxide, could be destructive, as its overproduction could destroy cell proteins, lipids, and DNA. H_2O_2 catabolism is under the control of glutahione peroxidase and catalase. Inhibition of H_2O_2 production is of importance in Alzheimer's disease, where the activity of SOD is increased and the activity of catalase is decreased. It was shown that in the presence of PRP the production of H_2O_2 induced by PMA was lowered (50%), as was SOD activity. No effect of PRP on catalase activity was observed. In the presence of PRP the activity of glutathione peroxidase was about 30% higher than in control blood samples.

The results obtained show that PRP can enhance glutathione peroxidase activity, an enzyme playing a role in the first line of antioxidant defense. This effect is sufficient to protect cells effectively against hydrogen peroxidase toxicity without catalase activation.

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

Malaria causes ca. 300-500 million clinical cases annually, the most severe form being caused by the parasite *Plasmodium falciparum* and responsible for the death of 2 million children per year. The invasion of erythrocytes by the malaria parasite involves several specific interactions. It was shown that glycophorins A, B, and C in the human erythrocyte membrane play a role as receptors for the *P. falciparum* antigens EBA-175 and EBA-140. The specificity of EBA-175 binding is dependent on the presence of sialic acid residues in glycophorin A O-glycan clusters. Glycophorin C (GPC), the minor erythrocyte glycophorin, appears to be a receptor for the parasite antigen BAEBL. Its binding to glycophorin C is dependent on sialic acid residues and on the peptide sequence. In order to elucidate this glycopeptidic receptor, we decided to investigate the structure of the N-glycan from GPC. As the first step of the procedure, a mixture of glycophorins (GPA, GPB and GPC) was applied to gel filtration on Sephadex G-200, but even in the presence of SDS the separation was not effective. Finally, a preparative gel electrophoresis appeared to be very effective in GPC isolation. The purity of GPC was confirmed by Western blotting and staining with anti-GPC and anti-GPA monoclonal antibodies. The N-glycosidic chain was released from GPC by enzymatic digestion; it will be analyzed by mass spectrometry in collaboration with Dr. V. Reinhold (USA).

We investigated the possibilities of the use of the surface plasmon resonance (SPR) technique in the analysis of protein glycosylation. This optical phenomenon is applied as a detection method in the biosensor BIAcore. Two sets of experiments were performed; the first involved analysis of the desialylation level of human glycophorin A (GPA). Three lectins were used: *Psathyrella velutina* (PVL), *Triticum vulgaris* (WGA), and *Sambucus nigra* (SNA-I); in experiments they served as the ligands. One of the lectins, SNA-I, showed a reaction with desialylated samples close to zero; the two other lectins exhibited some reaction due to the presence of recognizable GlcNAc residues in glycophorin N-glycans. Therefore, the better

reagent to determine residual sialic acid residues in GPA was shown to be SNA-I lectin. The second set of experiments considered the reaction of GPA samples isolated from blood groups A, B, and O erythrocytes with WGA lectin. This lectin recognizes Neu5Ac and GlcNAc residues in a glycotope. It was shown that reaction with WGA lectin was not equal for all samples: GPA-A exhibited the greatest reaction, GPA-B less, and GPA-O the least. These differences in reaction were evident and well documented. This finding is very interesting because the different reactions with WGA lectin suggest some differences in the investigated glycophorin samples, whereas published data on the carbohydrate moiety of glycophorin A describe no such the structural differences. Moreover, this result suggests that the SPR method may help in detecting very fine details in a carbohydrate moiety of an investigated glycopeptidic analyte, which makes the method more general in use.

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

Nur77 is an orphan nuclear receptor which is involved in several different intracellular processes, such as apoptosis, differentiation, and proliferation. Nur77 is implicated in the negative selection of autoreactive thymocytes, but its exact function is unknown. We showed that inducing Nur77 by ionomycin and its ability to bind DNA are not sufficient to initiate the apoptotic process in a thymic lymphoma cell line. Moreover, when apoptosis was restored by treatment with FK506, the NBRE-binding activity of Nur77 was decreased. More recently we showed for the first time that in normal thymocytes undergoing apoptosis, ionomycin induces translocation of endogenous Nur77 not only to the nucleus, but also to mitochondria. Immunosuppressant FK506 inhibits Nur77 NBRE and NurRE binding activity but has no effect on thymocytes apoptosis, the subcellular localization of Nur77, or cytochrome c release. This indicates that thymocytes can undergo apoptosis through the intrinsic Nur77-mediated mitochondrial pathway and that the transactivation activity of Nur77 monomers or dimers is not necessary for thymocyte apoptosis. This raises the possibility of a translocation-dependent role of Nur77 in thymic lymphoma cells. However, we showed that thymic lymphomas from mice with an autoreactive TCR are resistant to calcium-mediated apoptosis due to impairment

of cytochrome c release despite induction of Nur77 expression and its mitochondrial translocation. The impairment of cytochrome c release is sensitive to FK506. This indicates that the molecular target which overcomes lymphoma cells' resistance to apoptotic stimuli is located on mitochondria and could be regulated by calcineurin or the JNK/p38 pathway or both, as it was shown that these enzymes were inhibited by FK506. Our results provide new insight into the role of FK506-sensitive factors as a critical link between calcium signaling and resistance of lymphoma cells to death.

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

New strategies in experimental antitumor immunotherapy; Optimization of schedules of the dendritic cell-based vaccine application.

Murine dendritic cells (DCs) of the established JAWS II cell line as well as bone marrow-derived DC (BM-DCs) were used in three series of experiments. In the first study, JAWS II cells transduced with a retroviral vector carrying murine interleukin 12 (IL-12) genes (JAWS II/IL-12 cells) were used as a temporary source of IL-12 for the immunotherapy of C57BL/6 mice bearing transplantable murine colon carcinoma (MC38). The cell vaccines were administered according to different application schedules into the vicinity of subcutaneously growing palpable tumors. The JAWS II/IL-12 cells were delivered alone or in combination with JAWS II cells activated with MC38 tumor cell lysate (TAg; JAWS II/TAg cells). Mice treated with three consecutive injections of JAWS II/IL-12 or JAWS II/TAg cells responded with moderate tumor growth delay (up to 6.5 and 9.5 days, respectively). After the administration of the JAWS II/IL-12 and JAWS II/TAg cell combination, the time required for the tumor to reach a volume of 1 cm^3 was prolonged to up to 12.5 days. Increasing the number of DC-based vaccines to four resulted in an animal life-span extension of up to 87% over the control. The JAWS II/IL-12 cell vaccination of MC38 tumor-bearing mice was accompanied by an increased percentage of IFN-γ-producing $CD8^+$ spleen cells. In the second study, JAWS II cells transduced with EGFP gene or BM-DCs stained with intravital CFDA dye were loaded with TAg and then administered peritumorally to MC38 tumor-bearing C57BL/6 mice to analyze the migratory abilities of DCs applied as an anti-tumor vaccine and their capacity for immune response activation. On the 1st, 3rd, 5^{th}, and 7th days after injection the tumors, sentinel tumor-draining LNs and spleens were examined. The TAg-loaded DCs migrated more

effectively to the LNs than did the unloaded control DCs; however, the majority of them remained in the tumor vicinity. Immunohistological analysis of the tumor tissues demonstrated that only TAg-loaded DCs activated an immune response. Seven days after DC vaccine administration, numerous necrotic areas and some apoptotic bodies were observed in the tumor tissue. However, the anti-MC38 tumor cytotoxic activity of spleen and LN cells reached a maximum on the fifth day after DC injection. Concluding, TAg-loaded DCs migrated more efficiently to LNs and were more effective activators of local (but not systemic) cellular immune response than unloaded DCs. The third study was focused on the application of BM-DCs which were activated with T4 bacteriophages (T4 phages, T4) and further loaded with TAg in inducing an anti-tumor response. The activation of BM-DCs with T4 phages and TAg resulted in augmentation of their differentiation marker expression accompanied by an enhanced ability to prime T cells for IFN-γ production. The BMDC/T4 + TAg cells were used in experimental immunotherapy of C57BL/6 mice bearing advanced MC38 colon carcinoma tumors. As a result of their triple application, a significant tumor growth delay, up to 19 days, was observed compared with the controls treated with BM-DCs activated only with T4 phages, TAg, or lipopolysaccharide solution ["solvent"], where the tumor growth delay did not exceed 7 days. The percentage of tumor growth inhibition determined 10 days after the third cell injection ranged from 32% (for animals treated with BM-DC/TAg cells) to 76% (for animals treated with BM-DC/T4 + TAg cells) over the tumor-bearing untreated control mice. The obtained data indicate that *in vitro* interactions between T4 phages and BM-DCs followed by TAg activation caused augmentation of the anti-tumor effect when DCs were used as a vaccine for the treatment of tumor-bearing mice.

Toxicity and antitumor activity of the vitamin D analogs PRI-1906 and PRI-1907 in combined treatment with cytostatics

Active and less toxic vitamin D analogs could be useful for clinical applications. In the present study we evaluated the toxicity and antitumor effect of two new synthetic analogs of vitamin D, namely PRI-1906 ((24*E*)-24a-Homo-(1*S*)-1,25-dihydroxyergocalciferol) and its side-chain unsaturated homologue PRI-1907. The toxicity and calcemic activity as well as the antitumor effect of calcitriol analogs was investigated *in vivo*. The studies were performed in a mouse mammary 16/C cancer model. Since calcitriol and its analogs inhibited 16/C tumor growth only slightly, we applied them in combined therapy with cyclophosphamide (CY). Moreover, cell cycle analysis and VDR and p27 expression were investigated. The LD_{50}

values after five daily s.c. injections were 7.8, 10.0, and 2.4 μg/kg/day for calcitriol, PRI-1906, and PRI-1907, respectively. The serum calcium level increased to 40%, 23%, and 63% over the control for these compounds. We also compared the antitumor activities of PRI-1906 and calcitriol and the previously studied PRI-2191 (1,24-dihydroxyvitamin D_3, tacalcitol). Statistically significant inhibition of tumor growth by calcitriol up to the eighth day was observed in all schedules applied. PRI-1906 inhibited tumor growth at doses of 1 and 5 μg/kg/day, and PRI-2191 only at a dose of 5 μg/kg/day. Addition of the vitamin D analogs increased the antitumor effect of cyclophosphamide. PRI-1906 exhibited toxicity more than PRI-2191 but less than calcitriol and its antitumor activity was similar to both PRI-2191 and calcitriol. This new analog seems to be a good candidate for a combined treatment of mammary cancer.

In order to evaluate the effect of combined application of cisplatin, imatinib, or docetaxel and new vitamin D analogs (PRI-1906 and PRI-2191 against the cells of two human SCCs lines (SCC-25 and FaDu), cytotoxic activity and the effect on cell cycle and apoptosis was determined. The synergistic or additive antiproliferative effect was observed for all cytostatics used after treatment of the FaDu cell line with calcitriol or its analogs. Only at the lowest dose was antagonism caused by the combination of calcitriol and docetaxel shown. FaDu cells treated with cytostatics and vitamin D analogs accumulated in the G0/G1 stage. A statistically significant decrease (2 times) in the percentage of apoptotic cells was observed only in the combination of imatinib and calcitriol or PRI-1906. On the other hand, when the SCC-25 cell line incubated with cisplatin and imatinib in combination with calcitriol or PRI-2191 (100 nM) was used, the quantitative method of Chou and Talalay indicated antagonism. At lower doses of calcitriol or PRI-2191 combined with imatinib, the synergistic effect was observed, but in combination with cisplatin or docetaxel, only weak additivity was detected. Moreover, a significant decrease (2 times) in the percentage of SCC-25 cells undergoing apoptosis induced by docetaxel, cisplatin, and imatinib was observed. The combination of all cytostatic drugs applied with PRI-1906 at all doses caused synergism or additivity. These results might indicate that PRI-1906 is more effective than calcitriol or PRI-2191 as a potential anticancer agent when used in combination therapy with cytostatic agents. According to our knowledge, this is the first observation of interaction between calcitriol or its analogs and imatinib.

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

Studies on the 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

Beside 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 EXPERIMENTAL THERAPY
Head: Professor Michał Zimecki, Ph.D.

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

Lack of association between CD28 gene polymorphism and multiple myeloma in Polish population

Immunophenotypic studies revealed that CD28, a T cell-restricted molecule, is expressed on malignant plasma cells and CD28 expression in multiple myeloma (MM) highly correlates with poor prognosis and disease progression, while in normal plasma cells CD28 is always negative or only weakly positive in small subsets of plasma cells. It has been considered that predisposing genetic factors might be implicated in the disease. The study was undertaken to evaluate the association between *CD28* c.17+3T>C gene polymorphism with susceptibility to multiple myeloma in a Polish population, age of onset, and clinical course of the disease. One hundred fifty patients with MM and 243 healthy subjects were examined. The T>C transition at position 17 in intron 3 of *CD28* gene was genotyped by polymerase chain reaction followed by labeling with a SNaPshot kit and detection using a capillary genetic analyzer. The genotype, allele, and phenotype frequencies did not significantly differ in MM patients

compared with controls. No association between age of onset and clinical course of the disease and *CD28* gene polymorphism was found. The present study was unable to reveal any association between *CD28* c.17+3T>C gene polymorphism and MM, age of onset, or course of the disease.

Impaired zeta chain expression and IFN-gamma production in peripheral blood T and NK cells in patients with lung cancer in advanced stages of disease

Functional defects in T and NK cells from cancer patients have often been correlated with decreased expression of zeta chain. The zeta chain plays a crucial role in signal transducing events leading to T and NK cell activation, proliferation, and cytokine production. No data on zeta chain expression in patients with small-cell and non-small-cell lung cancer (SCLC and NSCLC) in advanced stages of disease have been reported so far. In our study we examined zeta chain expression in peripheral blood CD3+ and CD56+ cells and the capacity of these cells to produce IFN-gamma in correlation with the response to chemotherapy. Studies were performed in 15 patients with SCLC, 32 patients with NSCLC, and 17 controls. Before chemotherapy, the proportions of CD3+/zeta+, CD56+/zeta+, CD3+/IFN-gamma+, and CD56+/IFN-gamma+ did not differ among the SCLC and NSCLC patients and were markedly lower compared with controls. In the SCLC patients chemotherapy resulted in significant increases in the frequencies of CD56+/zeta+ lymphocytes, reaching the same values as in controls. In NSCLC patients no marked effect of chemotherapy on zeta chain expression was seen. In SCLC and NSCLC patients the chemotherapy resulted in significant decreases in the percentages of CD3+/IFN-gamma+ and CD56+/IFN-gamma+ cells. The frequencies of these cells among the lung cancer patients were comparable and remained markedly lower than those found in controls. Our findings suggest that impairment of zeta chain expression and IFN-gamma production in SCLC and NSCLC patients before and after chemotherapy may be one of the mechanisms leading to immune dysregulation in these diseases.

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

Studies on the immunotropic properties of lactoferrin (LF) were continued. LF is an iron-binding protein contained in secretory fluids and neutrophils of mammals. We used the *in*

vitro model of the secondary humoral immune response to sheep erythrocytes (SRBCs) suppressed by methotrexate (MTX). Previous experiments revealed a protective action of native, secretory LF on the immune response in that model. Subsequent investigations were aimed at evaluation of the biological activity of human recombinant LF (hrLF) produced by *Pichia pastoris* and equipped with a sugar moiety identical to that of native, neutrophil-derived human LF (source: Dr. M. Kruzel, University of Texas, Houston). We demonstrated that hrLF counteracted the suppressive action of MTX and identified its cellular receptor as sialoadhesin. Studies are underway to determine the activity of hrLF in other experimental *in vitro* models.

Investigations were also conducted on the VPRSGEVYT fragment of the HLA-DR molecule, localized on its exposed loop. New dimeric analogs were constructed (Prof. Z. Szewczuk, Wroclaw University), where V-T peptides were connected by their N-termini by means of polyethyl linkers of different length which were able to mimic the dimeric nature of the immunosuppressive fragments of HLA class II molecules. The results showed that the dimerization of the peptides led to increased suppressive activity which was dependent on the linker length.

Studies were continued on the immunotropic activities of the isoxazole derivatives of potential application in therapy. New lead structures were synthesized (Dr. S. Ryng, Wroclaw Medical University). The compounds were tested in several models, such as the secondary humoral immune response to SRBCs *in vitro*, the proliferative response of splenocytes to mitogens, and lipopolysaccharide (LPS)-induced cytokine production (TNFalpha and IL-6). The four studied compounds displayed very individual activity profiles in the applied tests (mainly immunosuppressive).

In the studies on cyclolinopeptide (CLA) we demonstrated that modifications of its structure (Prof. Zabrocki, £ódŸ Technical University) may lead to the acquirement of interesting new properties of therapeutic value. First, it appeared that CLA may exhibit opposite actions depending on the dose used. A high dose was inhibitory, a low one stimulatory, whereas a “medium” concentration was without effect. However, when CLA was used with MTX, an additive suppressive effect was observed, even at the lowest CLA dose. Such a result may have important potential value, since it creates a possibility of applying low (nontoxic) MTX doses when combined with CLA.

In the studies devoted to experimental phage therapy we demonstrated that pretreatment of mice with LF (both oral and intraperitoneal) increased the ability to kill *Escherichia coli* and

Staphylococcus aureus in the livers when suboptimal numbers of specific phages were applied. Such results indicate that phage therapy may be aided by LF administration. In another type of study we showed that phages were effective in significantly reducing bacteria numbers in the organs of mice subjected to immunosuppression.

Bone formation and remodeling involves the recruitment of osteoprogenitor cells from bone marrow cells and their proliferation and terminal differentiation into active osteoblasts. Failure or reduced activity of one of these processes results in impaired skeletal growth or reduction of bone mass. The majority of information regarding the development of the osteogenic lineage has been obtained from *in vitro* or combined *in vitro* and *in vivo* studies that utilized animal-derived cells. In 2007 we examined the capability of NIH3T3 fibroblasts and fibroblasts isolated from mouse muscles to express osteoblastic markers following *in vitro* stimulation with a number of cytokines. The cytokines used (IL-1, IL-6, and TNF-α) were derived from the culture supernatants of the macrophage cell line RAW 264.7 stimulated with lipopolysaccharide. We found that the cytokines were able to stimulate the expressions of two investigated specific osteoblastic markers, RANKL and osteocalcin, in both studied types of cells. These data indicate that fibroblasts have the potential to adopt an osteoblast-like phenotype and may prove to be a convenient model for studying the early events of the osteogenic differentiation process *in vitro*.

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 the role of bacterial surface glycoconjugates and protein antigens in immune response

This laboratory is currently involved in studies on the mechanisms of pathogenicity of diseases with bacterial etiology, the roles of molecular mimicry, bacterial proteins, and glycolipids in pathogenicity, and the structure and functions of bacterial capsular antigens and endotoxins. Within the framework of the search for molecular markers of bacterial infections and prognostic factors, the investigation was focused on new methods for the detection of endotoxins (*J. Luminesc*., 2007, 122-123, 987-989). This approach relies on luminescence depolarization effects in protein-modified silica films doped with organic luminophores

applied in biosensor applications. The monitoring of specific markers for sepsis and septic shock could significantly facilitate the prognosis of these diseases and their treatment. The general strategy for developing protective tools against invading bacteria comprises determining the structures of molecules involved in an infection and the immune processes, their chemical and genetic manipulations, as well as understanding of their biological activities. In addition to a new method for the isolation of glycolipids, we showed that lipopolysaccharides could also be extracted using supercritical carbon dioxide (*J. Supercrit. Fluid*, 2007, in press; doi:10.1016/j.supflu.2007.11.011). Regarding bacterial glycolipids, these molecules appeared to be useful markers for the identification of clinical isolates, and in the framework of these studies a new genus was described, *Ruania albidiflava gen. nov.*, *sp. nov.*, a novel member of the suborder of *Micrococcineae* (*Int. J. Syst. Evol. Microbiol*. 2007, 57(Pt 4), 809-814). The study 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 to *Enterobacteriaceae* strains.

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

Study on nonspecific immunity in viral infection

Two activities of innate antiviral immunity were studied: the resistance of human peripheral blood mononuclear cells (PMBCs) *ex vivo* to viral infection and the production of cytokines. Samples of blood were taken from healthy blood donors and from persons with frequent infections of the upper respiratory system. PMBCs were isolated by gradient centrifugation. Vesicular stomatitis virus (VSV) was used as the indicatory virus to infect PMBCs. The cytokines IFN, TNF, and IL-6 were titrated by biological methods and IL-10 by ELISA. Blood donors were divided for two groups: those with VSV-resistant and those with VSV-sensitive PMBCs, and the secretion of cytokines by their PMBCs was compared. The resistant PMBCs produced more cytokines than the sensitive ones. A statistically significant difference was found only in the case of the IFNs. To examine the contribution of IFNs and TNF in maintaining resistance, leukocytes from both groups were treated with specific anti-cytokine antibodies. Our previous study showed that the elimination of spontaneous IFNα, IFNβ, IFNγ, and TNFα from resistant leukocytes resulted in increased VSV replication. This

indicates the important role of cytokines. In VSV-sensitive PMBCs, anti-IFNα showed the opposite effect (decreased viral replication). In the absence of spontaneous IFNα, disturbances in cytokine production were observed. It was concluded that complete resistance of PMBCs to VSV infection is accompanied by higher cytokine release. The paradoxical effect of anti-IFNα on virus replication in leukocytes sensitive to viral infection may be attributed to changes in the cytokine profile balance, i.e. high TNF production by VSV-infected leukocytes and complete reduction of IL-6 production. The results were published by Orzechowska et al., *Arch. Immunol. Ther. Exp*., 2007, 55,111-117

A further study concerned the structure-property relations and cytotoxicity of isosorbide-based biodegradable polyurethane scaffolds for tissue repair and regeneration. Microporous scaffolds with controllable porosity were produced from solutions of biodegradable aliphatic isosorbide-based polyurethane in high-boiling-point solvents using a combined salt-leaching solvent-evaporation-coagulation process. The use of highly alkaline sodium phosphate heptahydrate as a solid porogene for scaffold preparation did not affect the polymer's molecular weight, but, surprisingly, modified the scaffolds' surface characteristics. Thus the scaffolds' surface showed higher oxygen and nitrogen concentrations and lower hydrocarbon concentration than the surface of the control material. This enhanced the scaffolds' surface hydrophilicity. The MTT cytotoxicity assay has shown that the isosorbide-based polyurethane scaffolds are noncytotoxic. The amount of IL-6 and IL-8 proinflammatory cytokines released from human leukocytes incubated in the presence of the polyurethane scaffolds was comparable to that released in the presence of culture-grade polystyrene, which served as a (negative) control. The microporous scaffolds from new biodegradable isosorbide-based polyurethane are promising candidates for tissue repair and regeneration. The monitoring of the stimulation of these mediators could provide information about the potential early and late cell reactions to the new biomaterials and it could prove to be a sensitive test for the practical selection of materials. The results were published by Gogolewski S., Gorna K., Zaczynska E., Czarny A (2007).

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 segregation of bacterial chromosomes

Home page: www.iitd.pan.wroc.pl/dept/mic/index.htm

Alignment of multiple chromosomes along helical ParA scaffolding in sporulating Streptomyces hyphae

Streptomyces, 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. The conversion of multigenomic aerial hyphae of the mycelial organism *Streptomyces coelicolor* into chains of unigenomic spores requires the synchronous segregation of multiple chromosomes, providing an unusual context for chromosome segregation. The dynamic, mitosis-like segregation of bacterial chromosomes and plasmids often involves proteins of the ParA (ATPase) and ParB (DNA-binding protein) families. In *Streptomyces*, correct spatial organization of the *oriC*-proximal region prior to septum formation is achieved by the assembly of ParB into segregation complexes. We focused on the contribution of ParA to sporulation-associated chromosome segregation. Elimination of ParA strongly affects not only chromosome segregation, but also septation. In wild-type hyphae about to undergo sporulation, immunostained ParA was observed as a stretched double-helical filament, which accompanies the formation of ParB foci. We showed that ParA mediates efficient assembly of ParB complexes *in vivo* and *in vitro* and that ATP binding is crucial for ParA dimerization and interaction with ParB, but not for ParA localization *in vivo*. We suggest that *S. coelicolor* ParA provides scaffolding for the proper distribution of ParB complexes and consequently controls the synchronized segregation of several dozens of chromosomes, possibly mediating a segregation and septation checkpoint.

Dr. Dagmara Jakimowicz

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

It is known that cyclic nucleotides (cGMP and cAMP) affect the synthesis of inflammatory mediators, but less is known about the mechanisms of their action. We have continued studies on the role of cGMP/cAMP-dependent signaling pathways in the development of inflammatory process using the monocytic cell lines U937 and THP-1. It was determined that THP-1 cells express both soluble (sGC) and particulate (NPR-A) guanylyl cyclases, while U937 cells only express NPR-A. The differentiation of cells into macrophages caused changes in the expression of guanylyl cyclases. The activities of sGC and NPR-A increased in THP-1 cells, but the activity of NPR-A decreased in U937 cells. Both cell lines expressed cGMP-hydrolyzing or cGMP-dependent phosphodiesterases (PDEs) belonging to the PDE1, PDE3, and PDE9 families. U937 cells also expressed PDE2. Stimulation of sGC with nitric oxide donors or stimulation of NPR-A with atrial natriuretic peptide (ANP) in THP-1 cells did not affect the activity of the transcription factor NF-κB. At the same time the activity of NF-κB and AP-1 induced by LPS was significantly decreased in the presence of ANP. Similar effects were observed in the case of U937 cells. It was also found that in both cell lines the membrane-permeable analog of cAMP, 8Br-cAMP, caused a similar decrease in the NF-κB activity induced by LPS. Effects of ANP and 8Br-cAMP were abolished in the presence of an inhibitor of cAMP-dependent protein kinase (PKA), suggesting that a common cGMP/cAMP signaling pathway might be involved in this process. Further studies are projected to clarify this issue.

Recently, much emphasis is being placed on the search of alternative methods in the therapy of bacterial infections which are not susceptible to antibiotics. A very promising strategy is based on the use of bacteria-specific viruses termed bacteriophages (phages). However, to ensure that such an approach is safe, it is essential to examine whether phages influence functions of human cells. We examined the influence of the phages T4 and A5 on the activity of NF-κB in human peripheral blood mononuclear cells (PBMCs), the monocytic cells U937 and THP1, and Jurkat T cells. The effects of the phages were compared with those induced by human herpesvirus-1 (HHV-1). As shown by means of the electromobility shift assay (EMSA), T4 and A5 phages did not change, while HHV-1 significantly elevated NF-κB activity in the analyzed cells. Interestingly, cells treated first with phages and then with HHV-

1 showed lower NF-κB activity than cells treated exclusively with HHV-1. These results indicate that phages not only do not stimulate the activity of NF-κB, but can also inhibit that activity stimulated by other viruses.

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

Laboratory of Tissue Immunology
Acting head: Assistant Professor Beata Nowakowska, Ph.D.

The association of HLA class II genes with immunopathogenesis of cryptorchidism

Cryptorchidism is one of the commonest congenital anomalies in childhood. It is a cause of male infertility and increases the risk of testicular cancer. Further investigation demonstrated that some of HLA class I antigens were associated with the pathogenesis of cryptochorchidism. In our study, clinical heterogeneity, the production of antisperm antibody, and familial occurrence were investigated to verify genetic associations with HLA class II alleles. Fifty-eight boys from the Lviv region (Ukraine) with unilateral and bilateral cryptorchidism were studied. We noted a significant association of HLA alleles with the production of antisperm antibody. The frequency of HLA DQB1*06 was higher in the group with antibodies (n=19) than without them (n=36) (chi-squared p=0.0151). Moreover, the HLA DRB1*04 allele was observed significantly more rarely in the group with antisperm antibodies (p=0.0470). The HLA DRB1*11 allele seems to be related with familial history of disease (p=0.0015). HLA class II alleles were not implicated in the monolateral or bilateral forms of the disease.

Genetic predisposition to cancer development

Germline p53 mutations are associated with Li-Fraumeni syndrome (LFS) and Li-Fraumeni-like syndrome. In a search for germline p53 mutations, a new series of cancer families with LFS or related cancer phenotypes was studied. A novel germline p53 mutation, discovered in a child diagnosed with a synovial sarcoma at the age of 8 years and an osteosarcoma at 12, was a missense CGA>CCA mutation at codon 342, which caused a substitution of proline for arginine. The second germline p53 mutation was discovered in a boy with a lung metastasis of adrenocortical carcinoma. The mutation was also at codon 342 (CGA>TGA), generating a change from arginine to a stop codon. Another p53 defect was

found in a child from an LFS family previously treated for rhabdomyosarcoma and osteosarcoma who had been recently diagnosed with MDS (AML). The alteration appeared to be a new p53 somatic mutation detected only at the MDS (AML) stage. The heterozygous mutation was in exon 10 codon 337 (CGC>GGC), resulting in a substitution of glycine for arginine. The defect was confirmed at the RNA level, where only the mutant Gly-337 was observed.

In summary, in this new series of cancer families, two germline p53 mutations were found, R342X and the new R342P, and, in addition, the new somatic mutation R337G. It is of note that in the Polish population, where constitutional p53 mutations are very rare, the two reported germline mutations were found in the tetramerization domain and the new somatic mutation as well.

Genetic polymorphisms and ovarian cancer

DNA sequence variation may explain some of interindividual differences in drug-induced adverse reactions and therapeutic responses. Genes which may potentially have an impact on risk of ovarian cancer development or on a patients' response to chemotherapy comprise the genes of the pathways of xenobiotic metabolism, DNA repair, and ABC transporters. In a case-control study we have investigated single-nucleotide polymorphisms (SNPs) in genes of the above-mentioned classes. The frequencies of the studied variant alleles and genotypes were similar in ovarian cancer patients and in the control group except for the XPC gene. In the XPC SNPs Liz939Gln A>C and intron 11 -5 C>A, the variant alleles and genotypes showed a prospective effect (p=0.025) and the observed *ORs* were, respectively, 0.69 (95%*CI*: 0.50-0.96) and 0.45 (95%*CI*: 0.22-0.91).

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

Investigations of ER-alpha expression in immune cells of male C3H/He mice in response to syngeneic antigen

Immune responses against auto- and syngenic antigens leading to the development of autoimmune diseases may be influenced by estrogens. The involvement of this hormones in the control of immunological reactions results from their direct influence on lymphocytes

through the presence of classical and functional ER-alpha in these cells. The aim of the study was to examine the possible role of ER-alpha in the development of the immune response against TGC. C3H/He adult male mice were immunized s.c. with TGC without adjuvant. One group of immunized mice was treated orally with tamoxifen. Immune response was controlled by assessment of the level of specific antibodies and DTH response. Forty days after the first immunization, the relative level of ER-alpha protein was measured in splenocytes (CD3CD4, CD3CD8, CD3, CD19) and thymocytes (CD4, CD8, CD4CD8) by the tri-color staining cytometric method. Total RNA was extracted from spleen and thymus cells and reverse transcribed in the presence of the gene-specific primers. cDNA templates were amplified in real-time PCR (SYBR Green) using a pair of specific primers encompassing exons 7 and 8. One band of the expected size was revealed using ER-alpha-specific primers. Immunization of animals with TGC antigens resulted in significantly increased levels of specific antibodies and DTH response and a higher relative level of ER-alpha in CD3, CD3CD4, and CD19 splenocytes with concomitant increased ER-alpha mRNA levels in splenocytes. In thymocytes the level of ER-alpha was increased in double-positive and CD4+CD8- cells. Mice treated with tamoxifen and immunized with TGC showed a lower relative level of ER-alpha protein in splenocytes. Some of the immunized and tamoxifen-treated animals had higher levels of anti-TGC antigens and higher DTH response. The results indicate that estrogens may have modulatory influence on the immune response against syngenic TGC and may play a role in the development of immune-related infertility in males.

Investigations on peripheral lymphocyte activity in women with endometriosis

Differential biological behavior of peripheral T cells, NK cells, and monocytes in women with endometriosis in contrast to those without this disease are observed. On the other hand, outcomes trying to find any correlation between cytokine expression and the behavior of immune cells connected to endometriosis development at the systemic level remain unresolved. The subject of this study was an investigation of the cytokines known to be important in the course of endometriosis: tumor necrosis factor-alpha (TNF-alpha), interferon-gamma (IFN-gamma), interleukin-8 (IL-8), monocyte chemotactic protein-1 (MCP-1), interleukin-6 (IL-6), and interleukin-10 (IL-10). We suspected that the production of cytokines could be differential between T cells and monocytes in women with endometriosis. For this reason in this study we investigated *in vitro* intracellular cytokine production by stimulated

peripheral T cells and monocytes derived from women with endometriosis and compared it with those with uterine leiomyoma, adenomyosis, and healthy women. We showed that the intracellular production of IFN-gamma was significantly decreased in advanced endometriosis patients in the CD4+ population of lymphocytes compared with women with mild endometriosis and control groups. In turn, the production of IL-8 was significantly increased in the CD8+ lymphocytes of women with mild and the CD4+ and CD8+ lymphocytes of women with advanced stages of endometriosis. Within the CD14+ cell population a significant increase in the production of MCP-1 was noted in women with advanced endometriosis and adenomyosis compared with that of women with mild endometriosis and the remaining control patients. In addition we observed a significantly increased production of IL-10 in advanced endometriosis compared with the mild fom.

In conclusion, Th1 type response could be decreased due to disorders in IFN-gamma production and polarization toward Th2 type response is probably enhanced by increased MCP-1 production in women with endometriosis.

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

The variable outcome of cancer patients with similar clinical status creates a need to search for reliable new prognostic indicators of tumor progression, recurrence, and survival. The aim of the study was to determine the prognostic value of new molecular traits of tumor cells in breast cancer which could determine tumor progression and metastatic growth. It has been hypothesized that tumor cells expressing FasL (Fas death receptor ligand) are able to avoid immune surveillance. The immunohistochemical semi-quantitative evaluation of the expression levels of Fas/FasL, p53 protein, survivin, and the vascular growth factors VEGF-C and VEGF-D in the tumor tissue of 130 patients of the Lower Silesian Oncology Center with stage II breast carcinoma was performed with the collaboration with Dr. M. Bêbenek and the group of Prof. J. Kornafel. The results, analyzed after five and ten years of follow-up, indeed indicated that Fas presence on human breast cancer cells significantly correlated with a lower rate of lymph node involvement and bone metastasis and longer disease-free time and survival.

Endothelial cells are critical in the recruitment and migration of circulating effector cells into sites of inflammation and necrosis. However, during the metastatic spread of tumor cells, extravasation from blood vessels is also a prerequisite for distant tissue colonization. Our studies are aimed at endothelial cells and, particularly, cancer cell metastasis which involves close adhesive interactions of cancer cells to endothelial cells at the site of cancer cell extravasation. Human umbilical cord blood is a rich source of hematopoietic stem and progenitor cells, among which are endothelial precursor cells able to modulate postnatal neovascularization. The aim of the study was a detailed characterization of endothelial progenitor cells isolated from umbilical cord blood, particularly multidrug-resistance protein expression, postulated as a novel marker of stem and progenitor cells. Flow cytometric analysis of human CD34/CD133 double-positive cells isolated from umbilical cord blood revealed that cells characterized by simultaneous high expression of CD133 and CD34 antigens were also positive for CD31, CD117, and CD105 antigens. The majority of CD34/CD133 double-positive cells expressed VEGFR-2 (KDR/flk-1) and bound *Ulex europaeus* agglutinin-1. The cells were negative for the mature endothelial cell markers vWf and VE-cadherin. Analysis of the expressions of the MDR1, MDR3, MRP1, BCRP, and LRP proteins in the isolated CD34/CD133 double-positive endothelial progenitor cells using flow cytometry, immunocytochemistry, Rhodamine 123 efflux assay, and reverse transcription-PCR techniques revealed MDR1, MDR3, MRP1, and BCRP protein expression on the examined cells. The isolated cells did not express LRP protein. These observations suggest that at least some of the multidrug-resistance proteins could be used as a marker of early endothelial progenitor cells. They also provide strong evidence for the existence of different subpopulations of endothelial cell precursors in cord blood and imply the possibility of using *ex vivo* expanded cord blood endothelial precursor cells as a tool in the investigation of the postnatal lineage diversification.

The studies on endothelial cells were performed in collaboration with Dr. Claudine Kieda, CBM CNRS UPR 4301, Orleans, France.

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

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

CTLA-4 gene singl- nucleotide polymorphisms (SNPs) in psoriasis vulgaris

Psoriasis vulgaris is a multifactorial disease with an autoimmune component, and T lymphocytes seem to be involved in its etiology. CTLA-4 molecule is an important downregulator of T-lymphocyte activation, and several polymorphisms of the *CTLA-4* gene were found to be associated with some autoimmune diseases. We examined whether the single-nucleotide polymorphisms (SNPs) CT60A>G and +49A>G in the *CTLA-4* gene are associated with psoriasis vulgaris. Alleles of these two SNPs were determined by the PCR-RFLP method. Both the CT60G>A and the +49A>G alleles and genotypes were distributed similarly in patients and controls. Although the two SNPs studied here in Poles were in linkage disequilibrium, all four possible two-locus haplotypes were found, one of them rare; of the remaining three, the haplotype +49G, CT60G was significantly (p=0.019, *OR*=0.58, 95%*CI*: 0.37-0.91) less frequent in the patient group with disease onset between the ages of 21 and 40 years than in controls and the other patient groups, whereas the frequencies of the other haplotypes were similar in patients and controls. To the authors' knowledge, this is the first study on *CTLA-4* CT60 allele frequencies in psoriasis [£uszczek et al., Int. J. Immunogenet. 2008; 35: 51-55].

KIR genes in complications of rheumatoid arthritis

We investigated whether killer-cell immunoglobulin-like receptor (*KIR*) genes are risk factor(s) for rheumatoid arthritis (RA) and its clinical manifestations. One hundred and seventy-seven RA patients and 243 healthy individuals were tested for the presence of 11 *KIR* genes using the PCR-SSP method. The frequencies of *KIRs* in patients with RA were similar to those in controls. However, RA patients positive for *KIR2DL3* and negative for *KIR2DS3* had earlier disease diagnosis. Additionally, *KIR2DL2* and *KIR2DS2* were significantly more frequent among RA patients with extra-articular manifestations and in its subgroup with vasculitis than in controls and in patients without these complications. Furthermore, the frequencies of *KIR2DS1* and *KIR3DS1* were lower in patients without bone erosions compared with healthy individuals. The relationships between the presence or absence of autoantibodies (rheumatoid factor and anti-cyclic citrullinated peptide) and *KIR* frequencies

were also evaluated, but no significant differences were observed. These results suggest that particular clinical manifestations of RA may have different genetic backgrounds with respect to *KIR* genotype (Majorczyk et al., *Genes Immun*., 2007; 8: 678-683).

Lack of association of PTPN22 single-nucleotide polymorphism with methotrexate treatment outcome in rheumatoid arthritis

PTPN22 1858C>T single-nucleotide polymorphism (SNP) is one of the major genetic risk factors of rheumatoid arthritis (RA). However, its role in the response of RA patients to therapy is not known. We examined a possible association of this SNP with the response of 169 RA patients to methotrexate (MTX) treatment. Rheumatoid arthritis was diagnosed according to the American College of Rheumatology (ACR) criteria. All patients were treated with MTX. Clinical improvement was evaluated according to the ACR 20% response criteria. DNA was isolated from venous blood and the 1858C>T SNP was established by polymerase chain reaction followed by restriction fragment length polymorphism using *Xcm*I digestion. Eighty-one patients responded to MTX with remission of symptoms, whereas 88 were nonresponders. Although 83.3% of patients with 1858TT genotype responded to MTX in contrast to 41.8% and 49.1% in CT and CC genotype bearers, respectively, this difference was not significant ($p>0.05$) due to the very low numbers of TT homozygotes in both groups of patients. Thus the response of RA patients to MTX treatment does not seem to depend on *PTPN22* 1858C>T SNP.

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