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Research Report – 2005

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

The orphan nuclear receptor Nur77 is a member of the Nur77 family belonging to the nuclear hormone receptor superfamily of transcription factors. The Nur77 protein (also termed NGFI-B, TR3, NR4A1) is a mediator of the cellular response to various external stimuli, leading to the proliferation, differentiation, apoptosis, and synthesis of steroid hormones, depending on the cell type.

Previously we showed that thymic lymphomas developing spontaneously in mice with transgenic TCR are resistant to TCR-induced calcium-mediated apoptosis. This blockade is localized downstream from the induction of Nur77 expression and upstream from the execution phase of apoptosis. Treatment with FK506 (inhibitor of calcineurin) or HA1004 (inhibitor of serine-threonine kinases) restored the sensitivity of these cells to ionomycin-induced apoptosis. We intend to explore the molecular mechanism of Nur77 action in normal thymocytes as well as the lack of its activity in transformed T cells. We found that in apoptosis-resistant cells, inomycin-induced Nur77 strongly binds DNA during the first two hours of response, in contrast to lymphoma cells treated with ionomycin together with FK506 or HA1004, which undergo massive apoptosis. We showed that Nur77 could discriminate between calcium signals sensitive to FK506 and those sensitive to HA1004, as the inhibitors differentially regulate the kinetics of Nur77 nuclear import, and FK506, unlike HA1004, inhibits Nur77 DNA-binding activity. In the presence of HA1004, NBRE binding by Nur77 protein increases with time (6 h vs. 2 h), whereas the final outcome of both inhibitors is apoptosis of thymic lymphoma cells.

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

Bordetella pertussis, an aerobic Gram-negative rod-like bacteria, is the causative agent of whooping cough (pertussis) in humans. Pertussis is a highly contagious disease involving the respiratory tract, especially dangerous for infants and young children. One of the virulence determinants of *B. pertussis* and the most abundant surface molecule is lipopolysaccharide (LPS). It plays a major role in host-pathogen interactions and is responsible for endotoxic activities. *B. pertussis* produces only two types of LPS, built of a lipid A moiety linked to a core nonasaccharide or a dodecasaccharide. The LPS of *B. pertussis* is devoid of an O-specific polysaccharide chain, represented instead by a single distal trisaccharide, thus structurally constituting a lipooligosaccharide (LOS). It was of interest to evaluate LOS epitopes as a target for antibodies that might be protective against biological activities of *B. pertussis*. We chose for these experiments the LOS of *B. pertussis* strain 186, i.e. the wild strain and the component of the whole-cell pertussis vaccine which has been used in Poland since 1978.

We determined the structural details of the *B. pertussis* strain 186 LOS and the anti-endotoxin activity of the polyclonal antibodies against the covalent conjugate of the core terminal pentasaccharide with tetanus toxoid. Antineoglycoconjugate antibodies inhibited the secretion of TNFα, IL-6 and NO by LOS-stimulated J774A.1 cells. We also performed STD NMR experiments for the identification of the binding epitope within the investigated pentasaccharide. We found that it is located predominantly in the distal trisacharide of the endotoxin. Additionally, by using HR-MAS NMR analysis we were also able to identify the pentasaccharide directly on intact LOS and distinguish the LOS of *B. pertussis* strain 186 possessing a core dodecasaccharide from that of strain 606 possessing a core nonasaccharide.

Thus the discovery of a simple method to distinguish between a protective and a non-protective immune response to *B. pertussis* infection and defining the additional components of acellular pertussis vaccine seems very important. We anticipate that it could improve the

efficiency of the vaccine by promoting immune defense that can kill the bacteria and neutralize the harmful effects of the endotoxin.

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

MBP and PLP are the major components of myelin sheaths, accounting for about 30 and 50% of total myelin proteins, respectively. Human MBP exists in four main isoforms (17.3, 18.5, 20.2, 21.5 kDa) generated by alternative splicing. PLP is a highly hydrophobic integral membrane protein of oligodendrocytes, and its two isoforms, encoded by one gene, are generated by alternative splicing: a 26.6 kDa classic form and a 23.5 kDa DM20 protein. MBP and PLP are the most extensively studied candidate auto-antigens in multiple sclerosis (MS). We aimed at the isolation and characterization of CHO cell clones with stable expression of recombinant MBP or PLP. The rationale for using a mammalian system is the posttranslational modification of the recombinant proteins expressed in CHO cells. The obtained clones could be a very useful tool for characterizing the immune response in MS. Plasmids containing cDNA coding for MBP (21.5 kDa) and PLP (26 kDa) were gifts from Dr. A. Campagnoni (UCLA, LA, USA). cDNAs were transfected into CHO cells using FUGENE 6 reagent. Several cell clones were isolated by limiting dilution and characterized by flow cytometry using anti-MBP or anti-PLP monoclonal antibodies. Two CHO cell clones, A1 and C1, expressing recombinant MBP and PLP, respectively, were further analyzed. After fixation and permeabilization allowing intracellular antigen staining, the cells were observed for immunofluorescence in a confocal scanning microscope. Anti-MBP staining of the A1 clone produced extra-nuclear granules uniformly distributed throughout the entire cell body, indicating the cytoplasmic localization of the recombinant MBP. Anti-PLP staining of the C1 clone revealed punctate circles, reflecting the surface localization of the recombinant PLP. Both patterns of the stained CHO cells were consistent with the proper cellular localization of MBP and PLP observed in myelin sheaths. To further verify that MBP and PLP were expressed in the A1 and C1 clones, PCR analysis was performed using as a template cDNA reverse transcribed from the mRNA expressed in either clone. Bands of the predicted sizes, 794 bp for MBP and 528 bp for PLP, were obtained for the A1 and C1 clones, respectively; they were absent in an analysis of non-transfected CHO cells.

We have expressed and purified a chimeric protein, Fy^a-GPA, composed of the amino-terminal extracellular domain of the Duffy antigen (aa 3-60) and the C-terminal intracellular fragment of glycophorin A (GPA, aa 104-131). cDNA constructs encoding chimeric Duffy protein were cloned into two expression vectors: pComb3H and pGEX, and were expressed in bacteria. Proteins expressed in bacteria containing a hexahistydyl tag or glutathione-S-transferase (GST) tag were purified by affinity chromatography on Ni-NTA-agarose or on glutathione-agarose, respectively. Two forms of recombinant protein (Fy^a-GPA and Fy^b-GPA) were obtained containing two different tags: hexahistydyl or GST. Recombinant proteins containing Duffy epitopes may be very useful in the production and characterization of monoclonal antibodies.

Carcinoembryonic antigen (CEA) is a well-characterized human tumor-associated antigen overexpressed by adenocarcinomas, primarily of the colon, rectum, breast, and lung. CEA functions *in vitro* as a Ca^{2+} independent, homo/heterotypic intercellular adhesion molecule which may play a role in cancer progression and metastasis via promotion of the aggregation of human colorectal carcinoma cells to liver and lung. CEA and CEA-related glycoproteins belong to the immunoglobulin superfamily (IgSF), which comprises a set of proteins involved in cell surface recognition events. We focused on the CEA-oligomerization mechanism. Twelve different CEA recombinant fragments, ranging from 80 (CEA/1-80) to 120 (CEA/1-100, -105, -110, -115, -116, -117, -118, -119 and -120) amino acids, were expressed in bacteria cells. We cloned cDNA of the CEA fragments into the pGEX vector which contains C-terminal His- and GST-tags. The recombinant CEA fragments were purified by affinity chromatography on agarose columns with immobilized Ni^{2+} (NTA-agarose) and glutathione-Sepharose. The degree of purification of the recombinant CEA fragments was evaluated electrophoretically (SDS-PAGE); the presence of CEA peptides was confirmed by immunoblotting with polyclonal anti-CEA antibodies. Our observations suggest that the CEA fragment 115-120aa is a potential inhibitor of homophilic CEA-dependent cell adhesion.

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

A proline-rich polypeptide complex (PRP) and its nonapeptide fragment (NP) did not induce reactive oxygen species secretion in human peripheral blood mononuclear cells (PBMCs). However, both PRP and NP, when applied to the cells simultaneously with PMA,

inhibited (40-60%) the H_2O_2-inducing activity of PMA in a dose-dependent manner. The inhibitory effect of PRP/NP on H_2O_2 secretion correlates very well with the inhibitory effect of the peptides on superoxide dismutase (SOD) activity in PBMCs. Many processes in the cell, e.g. the induction of cytokines and reactive oxygen species, are functionally connected with NF-κB. In the case of PBMCs treated both with PRP and NP simultaneously as well as 30 min before induction with LPS, 40% inhibition of NF-κB activity was observed. The results obtained suggests that the inhibitory effects of PRP and its active nonapeptide could be connected with the regulation of transcription processes.

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

Promoter analysis of the human α1,3/4-fucosyltransferase gene (FUT III)

In our previous studies we cloned and sequenced the promoter of human α1,3/4-fucosyltransferase. The presence of three binding sites for AP-1 transcription factor in the region representing the enhancer element of *FUT III* promoter suggested that this protein complex could be involved in the regulation of *FUT III* gene expression. To address this hypothesis, electrophoretic mobility assay (EMSA) was performed. It was found that oligonucleotides containing the AP-1 binding sequence corresponding to the region –672 to –710 from the translation initiation site in *FUT III* were bound by nuclear extracts from human colon carcinoma CX-1.1 cells. This observation was further confirmed by competition analysis with unlabeled oligonucleotides and antibody recognition assay with anti-AP-1 antibody directed against the N-terminus of human c-Jun p39. In the latter case, the band with the slowest mobility was identified as AP-1 complex, based on the reaction with anti-c-Jun antibody. However, it was not supershifted completely, suggesting that it could contain Ap-1 family proteins other than c-*jun* transcription factor.

The promoter activity of *FUT III* gene and FUT3 mRNA expression was analyzed in colon carcinoma cell lines with different expression of sialyl Le^a using the prFUT3/S and prFUT3/L constructs. A strong correlation between luciferase activity and mRNA level and the presence of the sialyl Le^a was found in the analyzed cell lines. These results support earlier data on the relations between the expression of FUT3 and the amount of this carbohydrate structure.

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

Initiation of bacterial chromosome replication

Bacterial chromosome replication is mediated by a single initiator protein, DnaA, that interacts specifically with multiple DnaA boxes located within the origin (*oriC*). *Streptomycetes* are Gram-positive mycelial soil bacteria with unique cell division features; they grow as substrate mycelia which differentiate to aerial mycelium and spores. The compartments of vegetative hyphae contain several copies of uncondensed chromosomes between occasional cross-walls, while the syncytial aerial hyphal tips may contain more than 50 copies of the chromosome. In *Streptomyces coelicolor*, replication is initiated by the DnaA protein at the centrally located *oriC* region and proceeds bidirectionally until the replication forks reach the ends of the linear chromosome. Most studies on the regulation of chromosome replication have focused on unicellular rod-shaped bacteria (dividing by binary fission), particularly on *E. coli*, with a single circular chromosome. The obvious differences between these bacteria and filamentous *Streptomyces* containing elongated compartments with multiple copies of a linear chromosome may imply differences in the regulation of chromosome replication. We identified three clusters of DnaA boxes (H24, H69, and D78) which lie within a relatively short segment of chromosome centered on the *oriC* region. Among the analyzed clusters, D78 exhibits the highest affinity toward the DnaA protein; the affinity of DnaA for the D78 cluster is about fivefold higher than that for *oriC.* The high-affinity DnaA boxes appear to be involved in the stringent control of chromosome replication particularly during the maturation of aerial mycelium. Deletion of D78 causes more frequent chromosome replication (an elevated ratio of origins to chromosome ends was observed) and earlier colony maturation. In contrast, delivery of extra copies of D78 causes slow colony growth, presumably as a consequence of a reduction in the initiation frequency of chromosome replication. We speculate that the number of high-affinity DnaA boxes is relatively constant within hyphal compartments and that the deletion of D78 therefore permits an increased copy number of either the chromosomal origin region or a plasmid harboring the D78 cluster.

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

We have continued studies on the regulatory role of cGMP-dependent signaling pathways in the activity of NF-κB and AP-1 in human peripheral blood mononuclear cells (PBMCs) and in rat peritoneal macrophages (rPMs). In PBMCs the elevation of cGMP resulted in activation of both transcription factors. This stimulatory effect of cGMP was additionally supported by the observed increase in IL-6 and TNF-α expression. On the other hand, in rPMs, which do not express cGMP-dependent protein kinase (PKG), induction of cGMP did not influence the NF-κB activity, but caused an elevation in AP-1 activity. Taken together, our results indicate that cGMP use different signaling pathways in the regulation of activity of each transcription factor.

DEPARTMENT OF EXPERIMENTAL ONCOLOGY
Head: Associate Professor Adam Opolski, Ph.D.

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

Correlation between the VDR expression and antiproliferative activity of vitamin D_3 compounds in combination with cytostatics

Calcitriol is a potent antiproliferative agent against various tumor cells *in vitro*. Its biological activity is mediated by the vitamin D receptors (VDRs). Here we present the results of study of vitamin D_3 compounds (calcitriol and its analogue PRI-2191) as potential agents in combined antitumor therapy *in vitro*. Applying anti-proliferative SRB and MTT assays, we measured the growth inhibitory effects of vitamin D compounds applied alone or in combination with either cisplatin or doxorubicin. Next we examined the correlation of this effect with the presence of nVDR (nuclear VDR). The following cancer cell lines were applied: HL-60 (human leukemia), SW707 (human colon cancer), A549 (human lung cancer), and WEHI-3 (mouse leukemia). We have shown that the treatment of tumor cells with the combination of vitamin D compounds and cytostatics decreased the 50% inhibitory concentration (IC_{50}) values compared with the effects of cytostatics applied alone. The synergistic effect was positively correlated with nVDR expression.

Studies on the mechanisms of MC38 colon carcinoma growth inhibition as the effects of experimental anti-tumor therapy with mouse dendritic cells (DCs) were continued

Our experiment proved that both the DCs of the JAWS II line loaded with lysate tumor antigen (JAWS II/TAg) and those transduced with IL-12 gene (JAWS II/IL-12) were able to migrate effectively to draining lymph nodes and activate immune response *in vivo.* The optimal effect was visible when mice were treated with JAWS/IL-12 cells. On the seventh day after vaccine administration, necrotic and apoptotic changes in tumor tissue as well as infiltrating lymphocytes were observed. In addition, spleen cell cytotoxicity towards MC38 cells gradually increased up to the seventh day. Three or four injections of mice with JAWS II/IL-12 cells, JAWS II/TAg, or both resulted in significant tumor growth inhibition. The most effective was the administration of JAWS II/IL-12 + JAWS II/TAg cells, which resulted in long-lasting, statistically significant tumor growth delay. Comparative studies on the anti-tumor effect of IL-12 or IL-2 transduced murine JAWS II cells vaccine were performed. Percentage differences in cells expressing maturation markers after efficient transduction with both SAMEN IL-12 and pQNIL-2 retroviral vectors were observed (CD86 down-regulation in the case of JAWS II/IL-2 cells). Despite the effective transduction of the JAWS II/IL-2 cells, no significant tumor delay was observed even if JAWS II/TAg+JAWS II/IL-2 cells were applied for vaccination. The T cell activation ability and anti-tumor activity of IL-12-transduced and tumor antigen-loaded mouse BM-DCs at different stages of maturation were also investigated.

Moreover, we positively answered the question whether bacteriophages support anti-tumor response initiated by DC-based vaccine in murine transplantable colon carcinoma We supposed that DC-phage interactions could intensify immune response in tumor-bearing mice. This phenomenon may be of potential use in anti-cancer immunotherapy. In collaboration with the Institute of Molecular Genetics of the Academy of Sciences of the Czech Republic, we conducted studies on the synergy of a CBM-4A ifosfamide derivative and IL-12 in mouse HPV 16-associated TC-1 tumor therapy.

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 at developing 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.
The mechanisms of immune deficiency in neoplastic diseases and autoimmune diseases

Recently, increased serum levels of the soluble form of CTLA-4 (sCTLA-4) generated by alternatively spliced CTLA-4 mRNA have been reported in several autoimmune diseases. This molecule is considered to be a new marker in these diseases. The purpose of the study was to investigate serum levels of sCTLA-4 and the effect of *ex vivo* stimulation with anti-CD3+rIL-2 on the production of sCTLA-4 by peripheral blood mononuclear cells (PBMCs) in patients with multiple sclerosis (MS) compared with normal subjects. Studies were performed in 37 patients with relapsing-remitting (RR) and 26 with the secondary progressive (SP) form of disease and in 24 age- and sex-matched healthy subjects. The serum and culture supernatant sCTLA-4 levels were estimated using ELISA (Bender, Germany). Patients with RRMS had significantly higher levels of sCTLA-4 in sera than patients with SPMS (p=0.006) and healthy controls (p=0.014). There was statistically higher production of sCTLA-4 by *ex vivo* stimulated PBMCs in RRMS and SPMS patients compared with normal subjects (p=0.001, p=0.001, respectively). In addition, spontaneous production of this molecule by PBMCs was observed in RRMS and SPMS patients. The levels of this molecule in culture supernatants were higher in RRMS patients (p=0.003) and SPMS (p=0.003) compared with normal controls. This is the first report showing increased sCTLA-4 serum levels in patients with RRMS and increased spontaneous and stimulated sCTLA-4 production by PBMCs in both groups of MS patients. The biological significance of sCTLA-4 in MS is unclear and requires further studies.

The pathogenesis of B-cell malignancies is poorly understood and associated with a disturbance in immune regulation. The CTLA-4 molecule plays an important role in immune regulation by downregulating the activation of T cells. Polymorphisms in the *CTLA-4* gene have been shown to be associated to a number of autoimmune diseases and lymphoproliferative disorders (multiple myeloma, non-Hodgkin's lymphoma). We analyzed three intragenic polymorphisms at the *CTLA-4* gene positions C-319T, A49G, and the dinucleotide (AT)n repeat polymorphism at position 642 in exon 3 in patients with B-CLL and in healthy controls. One hundred ten B-CLL patients and 105 healthy subjects were studied. Genomic DNA was isolated from whole frozen blood using the NucleoSpin^{R} Blood kit (MARCHEREY-NAGEL, Germany). 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 the fluorescence-based technique. The products were analyzed on an ABI PRISM 310 Genetic Analyzer (ABI PRISM 310 capillary electrophoresis system). Genotype analysis showed that the T allele at position -319 in the promoter region was over-represented among patients with B-CLL (P=0.0055, OR=2.10, 1.46<95%CI<2.91). No difference was observed for the A49G and the (AT)n polymorphisms (P>0.05). The three *CTLA-4* gene polymorphism C-319T, A49G, and 642(AT)n are in tight linkage disequilibrium with each other; therefore we extended the analysis to the haplotype distribution. The haplotypes -319C+49A$(AT)_8$ and -319C+49G$(AT)_{>8}$ were the most common both in patients and controls, with a frequency slightly higher in patients. No particular haplotype was significantly over-represented in affected or in healthy individuals. These findings indicate that the T allele at position -319 of the promoter region confers susceptibility to B-CLL in the Polish population.

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 on the adjuvant activity of the lactoferrin:monophosphoryl lipid A complex (LF:MPL) were continued. We demonstrated its adjuvant activity in the induction of both humoral as well as cellular immune response. An increase in specific antibody production was shown in the case of ovalbumin and *Plesiomonas shigelloides* immunization. Immunization of mice with *Plesiomonas shigelloides* together with LF:MPL complex protected the animals against *Plesiomonas*-induced sepsis.

Studies regarding the involvement of respective regions in the lactoferrin molecule in its immunotropic activities were also continued. For the *in vivo* and *in vitro* studies, native bovine and human LFs as well as recombinant human LF of plant origin were used. Both the native and recombinant LFs did not differ in their antigenic properties toward LF-specific T-cell lines. On the other hand, recombinant LF exhibited significantly lower adjuvant property than native LF. Since we had previously shown that the carbohydrate part of the LF molecule was essential for the adjuvant effect of LF, we concluded that a different composition of the glycan moiety in the recombinant LF (sugars typical for plant glycoproteins) cannot be recognized by antigen-presenting cells.

Other studies on milk-derived proteins demonstrated that glycomacropeptide, a derivative of kappa casein from bovine milk, exhibited protective activity in experimental endotoxemia and bacteremia.

In the course of our investigations on the protective action of bacteriophages in mice, we showed that oral administration of as few as 10^4 specific phage particles may be protective in animals intravenously infected with lethal doses of bacteria. In addition, lower (10^3) numbers of bacteriophages could act with LF in an additive manner in the clearance of bacteria from the organs of infected mice.

In vitro experiments revealed, in turn, that preincubation of phagocytes (human neutrophils or monocytes) with bacteriophages led to inhibition of phagocytosis, this process being observed both with homologous as well as heterologous bacteriophages. These results support earlier findings that bacteriophages can affect the metabolism of eucariotic cells. In contrast, preincubation of bacteria with homologous, but not heterologous, bacteriophages facilitated the phagocytosis, suggesting that specific opsonization of bacteria may be involved in that phenomenon.

A new method of isolating precursor osteoblast cells from human peripheral blood and determining their osteolytic activity was described. The cells could also be isolated from other species, such as guinea pig, rat, mouse, rabbit, and bovine. The technique is based on the determination of C-telopeptides, the degradation products of collagen from the bone during the osteolytic process. Mononuclear cells from the peripheral blood (PBMCs) acquire the osteolytic activity in the course of culture in vitamin D-containing medium in the presence of a substrate, i.e. a bone plate. Integrin receptors, which appear on immature precursor osteoblast cells derived from PBMCs, are responsible for this activity.

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

Laboratory of Transgenesis and Lymphocyte Biology
Head: Professor Paweł Kisielow, Ph.D.
NWC, a new evolutionarily conserved gene within RAG locus

Recently we have identified *NWC*, a new, evolutionarily conserved gene overlapping with the RAG locus. *NWC* is ubiquitously expressed, but in lymphocytes is differently regulated than in other cells. In developing lymphocytes it is dependent on the activity of RAG1 promoter and is transcribed together with RAG1 as a RAG1/*NWC* hybrid transcript, while in non-lymphocytes it is dependent on *NWC*'s own promoter located in the RAG2 intron, which in lymphocytes is silent.

This year we have identified hybrid RAG1/*NWC* transcripts also in human and chicken thymocytes, indicating that their discovery reveals a general, evolutionarily conserved phenomenon. Interestingly, two of the human hybrid transcripts, like the mouse hybrid transcripts, contain a novel 'RAG2 antisense' exon of a highly orthologous sequence. Together, these observations suggest that hybrid transcripts may have important biological functions.

In order to characterize factors responsible for the activation of the *NWC* promoter in non-lymphoid cells and its silencing in lymphoid cells, we identified the minimal *NWC* promoter. We found that the shortest fragment sufficient to activate the transcription of a reporter gene in DLR assay performed in non-lymphoid cells spanned the region from -119 to +125 nucleotides in relation to the non-hybrid *NWC* transcription start site. Sequence alignment of the characterized minimal promoter with the corresponding genomic sequences identified in other mammalian species (human, chimpanzee, rat, and dog) revealed the presence of two highly conserved motifs. Mutational analysis revealed that these fragments are necessary for the promoter activity. However, the *NWC* promoter fragments were also active in a DLR assay performed in lymphoid cells, suggesting that lymphoid cell-specific repression of the *NWC* promoter may result from the activity of a distal silencer and/or a factor repressing the transcription on the chromatin level.

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

Previously we observed binding of T4 phage to the membranes of cancer and normal blood cells. We selected a mutant, HAP1, with enhanced affinity for melanoma cells. Both T4 and HAP1 markedly and significantly inhibited experimental lung metastasis of murine B16 melanoma, and HAP1 was more effective than T4. We also found a mutation in the *hoc* gene that differentiates bacteriophage HAP1 and its parental strain T4: a non-sense type mutation that precludes proper synthesis of gpHoc. In our present work we proceeded with investigations of phage-eukaryotic cell interactions.

We compared the antitumor activities of bacteriophages after oral (*per os*) and intraperitoneal (i.p.) administration of lysates and purified preparations. Our observations indicate that *per os* application of a bacteriophage preparation is safer and at least as effective as i.p. Bacteriophages applied *per os* were more effective in inhibiting metastasis formation. These observations are of great importance in any consideration of possible therapeutic applications of bacteriophages.

We showed that T4 phage diminishes the luminol-dependent chemiluminescence of peripheral blood polymorphonuclear leukocytes stimulated by the lipopolysaccharide of *Escherichia coli*. The effect was also observed when live bacteria were used for stimulation and it was independent of the bacterial susceptibility to T4-mediated lysis. On the other hand, T4 did not influence ROS formation by polymononuclear leukocytes stimulated by PMA. These findings indicate that the elements of the mechanism enabling the phage to infect bacteria may not be involved in the observed phenomenon and that it is specific to some degree and may depend on the kind of activator. These observations provide new evidence for possible interactions between phages and mammalian cells.

In the course of our studies on the application of olfactory ensheathing cells (OECs) in the treatment of complete spinal cord injuries in humans, conducted in collaboration with the Department of Neurosurgery of the Wroclaw Medical University, we showed that the cadaver-derived olfactory bulb and olfactory mucosa can be considered a reliable source of OECs. Efficient culture of OECs from the cadaver olfactory bulb was possible when the warm

ischemia time was less than 20 minutes. In contrast, OECs derived from cadaver olfactory mucosa were resistant to warm ischemia lasting even 180 minutes. We also developed a simple procedure for short-term storage (up to 14 days) and partial purification of OECs by keeping them in nonadherent conditions. This enables grafting OECs without the necessity of using additional procedures connected with cell culture and purification (e.g. enzymatic detachment of cells, storage in liquid nitrogen).

Bacterial infections remain a major medical challenge in view of increasing antibiotic resistance among different bacterial strains. Coagulase-negative staphylococci (CoNS) have become the most often isolated bacteria from the blood cultures, spinal fluid, and respiratory tracts of neonates. These nosocomial strains are often resistant to many different groups of antibiotics. The sensitivity spectra of 50 CoNS strains isolated from neonatal bloodstream infections to antibiotics and to 23 specific staphylococcal bacteriophages were determined. Sixty percent of the multidrug-resistant staphylococci isolated from severe neonatal infections were sensitive to four bacteriophages, but most of these isolates exhibited high rates of resistance to vancomycin. The possible therapeutic use of phages was postulated.

Impact of specific bacteriophages on the properties of the sperm of boars infected by the blue pus bacterium Pseudomonas aeruginosa

The aim of the observations was to assess the influence of specific bacteriophages on the properties of the sperm of boars infected by the blue pus bacterium. The study covered 15 boar reproducers; 5 of them had been experimentally infected with *Pseudomonas aeruginosa,* whereas the remaining 10 were males in use at the Pig Insemination Station in whose preputial sac or sperm the microorganism concerned had been discovered. The therapy was pursued for three weeks. Before and after the treatment, sexual responses, sperm (quantity, concentration of spermatozoa, percentage of sperm cells having correct movement, their morphology and survival rate), peripheral blood, and the capacity of blood cells to produce cytokines were examined. No essential differences between the studied sexual responses before and after the therapy were found. Of the sperm properties, the only statistically significant differences were recorded for sperm cell survivability in the BTS dilutor, which increases after the treatment by 230-242%. After the therapy with bacteriophages, a decline was found in the level of IL-10 in blood serum and supernatants of cell cultures stimulated with LPS and those unstimulated. The results obtained (improvement of the survivability of spermatozoa) wholly justify the need to employ the bacteriophage therapy in the treatment of reproductive boars infected by the blue pus bacterium.

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 new and reliable 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 urinary bladder and breast cancer which could determine tumor progression and metastatic growth. The prognostic value of flow cytometric evaluation of DNA ploidy and tumor cell proliferative activity in surgical biopsies of kidney tumors were examined. In renal cancer, aneuploidy in combination with T>2 clinical stage and the presence of a high fraction of proliferating tumor cells indicated for increased probability of tumor progression. Soluble intercellular adhesion molecule-1 (sICAM-1) level was examined in renal cancer patients. It has been observed that preoperative high sICAM-1 level (<221 ng/ml) was connected with a high probability of post-operative disease progression and metastatic tumor growth. In breast cancer, the level of Fas/FasL expression in the tumor tissue of 130 patients with stage II breast carcinoma was evaluated. The results, analyzed after five years' follow-up, indicated that Fas presence on human breast cancer cells significantly correlated with the lower rate of lymph node involvement, longer disease-free time, and survival.

Our 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. Natural killer (NK) cells are innate effector cells that play a critical role in the early defense against viral infection and malignant transformation. On the other hand, ECs are critical in the recruitment and migration of circulating effector cells into sites of inflammation and necrosis. EC injury occurs in a variety of pathologic infections, autoimmunity, transplantation, and graft-vs.-host disease and may result from cytotoxicity toward ECs mediated by effector lymphocytes. We showed that organ-specific NK-mediated EC killing occurs in an IL-2 activation-dependent manner. Human NK cells were able to adhere and kill human endothelial cell cell lines. To decipher the way in which NK cells are recruited and bind the endothelial layer to enter the tissue underneath, we studied the adhesion mechanism of NK and ECs. Correlation was observed between the adhesion pattern and the susceptibility to NKL2-mediated killing. To identify the cytotoxic pathway used by NKL2 cells, the involvement of the classical and alternate pathways was

examined, indicating a predominant role of the perforin/granzyme pathway. The interaction between NKL2 effectors and ECs induced cytochrome *c* release and Bid translocation in target cells, indicating an involvement of the mitochondrial pathway in NKL2-induced EC death. The present studies emphasize that 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.

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

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

Cytokines in endometriosis

Endometriosis, a common disease in women of reproductive age is characterized by the presence of endometrial tissues outside the uterus. Dysmenorrhea, pelvic pain, and infertility are the main clinical manifestation of the disease. An immunological basis has been considered to be important in the pathogenesis of endometriosis. Chemokines are seen as one of the most important factors in the development of endometriotic implants. The aim of the study was to test the serum monocyte chemotactic protein level and to determine the population of immune cells secreting this chemokine. In women with endometriosis there was a significantly higher serum level of MCP-1 compared with healthy women and women with leiomyoma. In women with adenomiosis and advanced endometriosis there was a significantly higher number of CD14+ cells producing MCP-1 than in healthy women and women with leiomyoma.

Male infertility

Fibronectin has been found in tissues and fluids of the male reproductive tract. It is a component of spermatozoa, basement membrane of the testis, and epididymal and seminal fluid. The exact role of fibronectin in ejaculated semen is not known. The aim of the study was to identify degradation products of fibronectin in relation to the results of general semen examination. The relative amounts of 60, 90, and 100 kDa FN fragments were 2-3 times higher in seminal plasmas with abnormal semen characteristics than in a normozoospermic group.

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

The innate antiviral immunity of leukocytes in patients with chronic renal failure on hemodialysis was compared with that of a control group. The immunity was measured using the method of direct infection of peripheral blood leukocytes with indicatory VS (vesicular stomatitis) virus. The VS virus did not replicate in leukocytes with strong innate immunity, whereas by impaired immunity the virus multiplied to high titer. Patients on hemodialysis expressed the same levels of nonspecific antiviral immunity as the control group. This means that antiviral immunity was not impaired in chronic hemodialysed patients.

The activity of leukocytes from the lower respiratory tract of patients with lung inflammatory diseases with regard to coexisting viral or bacterial airway infections was studied. Assessment of the local inflammatory response in bronchial asthma patients indicate that the reduction in the activation of NFκB and the inhibition of production of TNFα and IL-6, but not IFNα and IFNβ, in pulmonary leukocytes may be a consequence of inhaled corticosteroid therapy. Analysis of the interrelationship between clinical status of the patients and the production of pro-inflammatory and antiviral cytokines revealed that IFNγ, which was produced only in the corticosteroid-treated patients, can involve, apart from IL-6 and TNFα, in deterioration of clinical symptoms.

There is increasing evidence to support the role of immune mechanisms in the pathogenesis and progression of chronic heart failure (CHF). Recent studies investigating signaling pathways responsible for the activation of the pro-inflammatory cytokine system in CHF revealed the role of nuclear factor kappa-B (NF-κB). The aim of study was therefore to evaluate the pattern of activation of the NF-κB system in peripheral blood leukocytes (PBLs) from CHF patients according to the severity and etiology of CHF and to relate it to clinical status, exercise intolerance, and inflammatory status. Additionally, in a series of *in vitro* experiments, the role of LPS as a stimulus for the NF-κB system in PBLs was evaluated. There is some evidence that in CHF patients, elevated LPS levels in peripheral blood can be a strong immune activator through action on circulating immunocompetent cells, with downstream activation of the NF-κB system as a signal transducer of immune response to LPS.

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

We continued the investigation on population genetic of HLA system. The aim of our study was to verify, by typing at the DNA level, the frequencies of the rare allele HLA locus B. A total of 325 unrelated, healthy individuals of both sexes living in the Wrocław area and in the neighboring districts were included in the study. Of the rare alleles we typed B*1503 (B72), B*1509 (B70), and B*1510 (B70) with a frequency of 0.006. We observed in our population the very rare alleles B*42 (recombinant B*07 and B*08) , B*54, and B*67 with a frequency of 0.0006

The HLA B*48, B*46, B*59, and B*53 alleles were not found in our study, although they are present in other caucasoid populations.

Genetic predisposition to cancer development

A polymorphism of p53 at codon 72 encoding an arginine (arg) or proline (pro) residue is located in a proline-rich region of p53 protein, a domain important for growth suppression and apoptosis. The two p53 variants have been reported to have different biochemical and functional properties. However, the significance of this polymorphism in respect to cancer risk is still uncertain. Strong correlation between the p53 codon 72 polymorphism and ethnicity is well known, and the arg allele is prevalent in populations living further away from the Equator. It is possible that the phenomenon of uneven allele distribution is further complicated by a differential allele expression in heterozygotes. We evaluated the expression status of codon 72 polymorphic alleles in healthy Polish (Caucasian) and Chinese (Asian) populations (collaboration with Dr K. Sabapathy, Laboratory of Molecular Carcinogenesis, National Cancer Center, Singapore) and in cancer patients. Preferential expression of the arg allele in healthy Polish heterozygote (arg/pro) subjects and the pro allele in Chinese heterozygotes (arg/pro) was observed. However, in the normal tissue surrounding the breast tumors of Chinese cancer patients, expression of the arginine instead of proline polymorph was observed. In the Polish patients, mostly with hematological neoplasms, the arginine polymorph seemed to be the preferentially expressed allele. The data point to the codon 72 expression status as a potential marker of an increased risk of cancer.

Genetic polymorphisms and ovarian cancer

Variations in DNA sequences may explain some of interindividual differences in drug-induced adverse reactions and therapeutic responses. Among the genes which potentially may

have an impact on patient response to chemotherapy are genes coding for drug-metabolizing enzymes, ABC transporters affecting both drug biodistibution and cancer sensitivity to chemotherapy, and members of DNA-repair pathways which might also influence cancer sensitivity to cytostatic agents. In a case-control study we investigated several single-nucleotide polymorphisms (SNP) in genes of the above-mentioned classes. In general, the frequencies of the studied variant alleles were similar in ovarian cancer patients and in the control group.

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

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

The cell surface receptor CTLA-4 is an important negative regulator of T cell activation. We hypothesized that polymorphisms of the *CTLA-4* gene influencing the gene expression, mRNA stability, or the intracellular transport of the CTLA-4 molecule may contribute to allergic asthma. Three single-nucleotide polymorphisms, -1147 C/T and -318 C/T in the promoter region and +49 A/G, which changes an amino acid in the leader peptide which affects the intracellular transport of the protein, and a microsatellite 642 $(AT)_n$ in the 3'-untranslated region, influencing mRNA stability, were tested. No statistically significant differences between patients and controls in any of these polymorphisms were found.

CTLA-4 gene polymorphisms and soluble CTLA-4 peripheral blood levels in psoriasis vulgaris

Psoriasis vulgaris is one of most frequent skin diseases in our population. It is an autoimmune disease of unknown etiology. We compared the frequencies of four CTLA-4 polymorphisms: -1147 C/ T, -318 C/T, +49, and 642 $(AT)_n$, in 116 patients with psoriasis vulgaris and 123 healthy donors. No statistically significant differences were found between the groups. However, levels of soluble CTLA-4 were significantly (p=0.022) higher in patients than in controls, and the difference was even more prominent (p=0.0055) for type I psoriasis (age at onset below 41 years). This result suggests that soluble CTLA-4 may be involved in the regulation of autoimmunity in psoriasis.

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

Current activity of this laboratory is focused on studies of the mechanisms of pathogenicity of diseases with bacterial etiology, the role of molecular mimicry, bacterial proteins, and glycolipids in pathogenicity, and the structure 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 was elaborated (*J. Microbiol. Meth*., 2006, 64, 171-184). The method relies on the determination of a chemical marker of endotoxin, 2-keto-3-deoxy-octulosonic acid, using GLC-MS (Kdo method). The specificity of the method covers a broad spectrum of endotoxins due to the enzymatic dephosphorylation of the interfering phosphate substituent of Kdo. The monitoring of specific markers for sepsis and septic shock could significantly facilitate the prognosis of these diseases and their treatment. Consequently, the second approach relies on the detection of another marker of endotoxin, 3-hydroxy fatty acids, which allow measurement of its level in patient sera (*Arch. Immunol. Ther. Exp*., 2005, in press). The general strategy for the elaboration of the protective tools against invading bacteria comprise the determination of the structures of the molecules involved in an infection and immune processes, their chemical and genetic manipulations, as well as understanding their biological activities. The interference of some bacterial components with functions of tissue structures may contribute to the mechanisms of pathogenicity. Due to structural mimicry, care should be taken when antibacterial vaccines are constructed to avoid the induction of autoantibodies. In this area we have identified an enolase-like 45-kDa outer-membrane protein which mimics human muscle enolase (*FEMS Immunol. Med. Microbiol*., 2005, 45, 53-62). Such a protein may be involved in pathogenicity. Another bacterial protein was found to be a useful carrier for safe and effective vaccines, namely fimbria (*FEMS Immunol. Med. Microbiol*., 2005, 45, 221-230).

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