PhD-theses

Immunomodulant mechanisms on the cellular level

written by

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Introduction

This dissertation is the result of more than 15 years' laboratory and clinical work of a paediatrician with an interest in genetics and immunology. The common in the apparently different topics is that the disturbance of the immune system was present in the background of all of them.
The following topics were investigated:

1. 55 minor anomalies were analysed in children with malignancies (as secondary immunodeficiency states) and in matched healthy controls.

2. Coding DNAs of immunomodulant proteins in common secondary immunodeficiency states (Cystic Fibrosis: CF, delta F 508 mutation and haematological disturbances) were detected by PCR based methods in order to introduce these techniques to everyday practice in Hungary.

3. We dealt with the immuno-modulant / immuno harnessing activity of several BRM (Biological Response Modifier) materials of bacterial, viral, liposomal origin as well as of other factors like age and gender. We also tried to apply the principle of optimal adjuvation (RAUSS: Immun-Forschg;'58,116:287) for liposomal vectors and liposome-lipid A complexes.

4. O'Connell et al published first that FasL+ colon carcinoma cells are able to induce apoptosis in Fas+ lymphocytes (the "Fas-counterattack mechanism " J.Exp. Med' 96,184:1075) We investigated this phenomenon on human melanoma cell lines (HMC).

5. We described acid sphingomyelinase (ASM) deficiency in a patient with Beckwith-Wiedemann syndrome (BWS) first in the literature. This special case was investigated further, as ASM is responsible for the production of ceramide, a key molecule of apoptosis.
We contributed to the recent, most comprehensive mapping of the human chromosome 11.

Materials and methods:

1. *Assessment of minor anomalies:* According to MÉHES et al: (Eur J. Ped. '85, 144: 243) *Investigated persons:* 100 children with tumors, 100 healthy matching controls. *Biometrics:* Chi -square
2. Pathologic/chimaera genes and their products in secondary immunodeficiences: A. *Delta F 508 mutation* screening: *DNA: Standard isolation* from peripheral blood. *PCR amplification:* ^{32}P *labelled primers*: CFTR VA-3 VA-4. Radioisotope: . Gel-*electrophoresis* on polyacrylamide gel, drying, autoradiography. B. *Diagnosis of chimaera genes*: mRNA isolation, cDNA synthesis according to standard methods. cDNA was used as template for further investigations.
3. *Factors influencing active immunization*: *Laboratory animals:* Mice: LATI-Swiss albino, A/NIMR/HU, CBA/NIMR/HU. Guinea pigs: Koppenhagen white guinea pigs. *Toxin-or virusneutralisation, haemagglutination-inhibition, ELISA:* acc. to standard methods (Hungarian Pharmacopoeia, Pharmacopoeia Internationalis [WHO], British Veterinary Codex. *Antigens used:* TCA-purified tetanus toxoid. BRM materials: killed anaerob corynebacteria: - C. parvum RR-1 -- C. acnes RR-2 - C. lymphophylum RR-33 - C. granulosum RR-H-5 - C. avidum RR-41. Brucella abortus (PH-0159) inactivated whole-cell susp. - Shigella flexneri (2a serotyp. - Shigella sonnei (I phase) -- Salmonella typhi: Ty 2 (Rauss-Lovrekovics strain). Bordetella pertussis and bronchiseptica. Germ-counts expressed in International Opacity Units (FDA*) Preparation of bacterial extracts:* According to slight modifications of standard methods. Endotoxins: sec. Boivin-Rauss, LPS: with ethanol precipitation . *Lipid-A sec. Kontrohr T, Pécs. Víruses:* NDV-H-/BLB 86, IBDV-BLB 90, AIBV-H-120-Mass, BRV PHX/81-82/1 *Tumor:* EHRLICH ascites tumor *(*Tumor-Bank of the National Institute of Oncology, Budapest): 10^8 viable cells/mouse**.** *Adjuvants:* $Al(OH)_3$, $AlPO_4$, complexometry. *Dosage:* acc. to "optimal adjuvancy" (Rauss-Kétyi-Réthy). *Liposomes, Liposome-Lipid A complexes*: acc. to Báthori and Kontrohr. *Liposomes*: lecithin, cholesterol, stearylamin: 8:1:1. *DDA-Cl (Arquad 2HT) source:* Wellcome Labs. (*Gall,* Beckenham, UK.) *Biometrics:* Computation of mean survival times, SD, kurtosis and skewness, Student's-t-test, Kolmogorov Smirnov analysis, ANOVA, MANOVA, khi ^{2} and generalized Wilcoxon test were estimated.

4. *HMC and TIL cell lines*: originated from surgical samples. Cell cultures were initiated in RPMI-1640 supplemeted with 15% FCS and AIM –V with 20% human LAK cell conditioned medium with 10 IU/ml rIL-2, respectively. *Immune histochemistry:* bcl-2, FasL and anti Fas (UBI and PharMingen) primary –and AP-conjugated anti-mouse secondary antibodies were used as recommended by the manufacturers. *Apoptosis* was assessed in Jurkat and TIL cell lines, with DNA-ladder, Annexin-V-binding and quantitatively by Boehringer-Mannheim Cell death ELISA kit.

5.*Measurement of ASM activity*: from skin fibroblasts, by standard in vitro assay. (VANIER: *Clin Genet* '85 27:20). *Diagnosis of BWS*: acc. to the criteria of ELLIOTT & MAHER (J. Med. Genet '94,31: 560) . *Comparison of SMPD1 and imprinted genes:* according to HURST et al (Nature Genet 1996;12:234-7). For the *elaboration of the model explaining the role of SMPD1 in BWS*, and for the *exact localisation of SMPD1 on chromosome 11* the clinical data of RÉTHY and MANNENS (Gyermekgyógyászat-Pediatrics, '98; 49: 358)**,** Med Pediatr Oncol '96;27:490; www-iag.unice.fr/workshop/SCW-11-6-Abstracts.html) were used. The (most relevant) *databases used for the integrated map* of chromosome 11 were: dbj.nig.ac.jp, gdbwww.gdb.org, genome-ww.stanford.edu

Results with original observations

1. The prevalence of minor anomalies in the patients' group (85%) was significantly higher than in the control group (66%) Some types of minor anomalies occurred more frequently in children with tumors than in healthy controls.

2. The PCR-based techniques for the detection of diseased genes and gene products in CF and in other secondary immunodeficiencies (malignant haematological disorders, bone marrow transplantations) and for the follow up of treatment were introduced by us among the firsts in Hungary. Pulmonary symptoms were more severe in patients with mutation delta F 508.

3. We proved the beneficial immunomodulant/ immuno-harnessing activity of several BRMs (of bacterial, viral and liposomal origin).
   According to our data, complete (3 shots of) active anti-tetanus immunization is necessary
   for mothers to prevent neonatal tetanus cases (i) and
   for babies to achieve full protection in the presence of circulating maternal antibodies (ii.).
   We published the first data
   -on the age and sex dependent differences of immunological memory and immune response both in humans and in guinea- pigs (i.),
   -on the beneficial effect of bacterial LPS/LipidA to diminish these differences(ii.).
   According to our results in Ehrlich-tumor infested mice,
   -pre-immunisation with therapeutical virus decreased the effectivity of oncolytic virus-therapy
   -Virus neutralisation could be prevented by liposomal entrapment (first presented in vivo data)
   -LPS/Lipid A enhanced the therapeutic activity of oncolytic viruses
   -The principle of optimal adjuvation concerns a broader range of BRMs including liposomal vectors and liposome/ lipid A complexes.

4. We were among the firsts who proved the mechanism of "Fas-counterattack" on HMC cell-lines.
   -We observed the simultaneous presence of Fas, FasL and Bcl-2 molecules on HMC lines first.
   -We published the hypothesis of the Fas-FasL autocrine loop that could be used by HMCs as a growth factor, in the presence of Bcl-2.
   -We were the first who published that FasL expression on HMCs may decrease/cease gradually upon in vitro cultivation.

5. -We published the deficiency of acidic sphingomyelinase (ASM) in Beckwith Wiedemann Syndrome (BWS) first in the literature.
   -Based on the analysis of the clinical case and the published experimental data (Ref: see Methodology) the following hypothesis was established: The gene responsible for ASM (SMPD1) is an imprinted, maternally expressed, BWS -and apoptisis-related growth suppressor gene.
   -We published the exact localization of SMPD1 on chromosome 11 (11p.15.4)

-We constructed and published the recent and most comprehensive map of human chromosome 11.

Discussion, Conclusions

1. Genes of organogenesis and their protein products involved in the genesis of minor anomalies may increase the risk of tumors. Our observations together with other investigations may help in the better understanding of the connections between prenatal mutations and carcinogenesis.

2. The frequency of delta F 508 mutation in Hungarian CF patients and carriers resembles the Middle-European values (66.15%). The leading symptoms of CF cases with delta F 508 mutations occurred mostly in the lower respiratory tract. Besides diagnostic perspectives molecular genetic methods may help to decide the necessity of supplementary immune-therapies in CF.

The detection of chimaeric genes and gene products proved to be a useful tool in bone –marrow transplantations (donor search, bone-marrow purging, cord-blood-screening), furthermore in the follow up of minimal residual leukaemias and/or the homing process of bone marrow transplants.

3. BRMs investigated by us are proved to be effective immune-modulators.

Neonatal tetanus is still a major factor of deaths in the Developing World. Our data on the necessity of complete active anti-tetanus immunisation of

-mothers (two plus one shots achieved 100% protection for both the mothers and their babies)
-babies for achieving 100% protection against tetanus in the presence of circulating maternal antibodies)

were internationally accepted to control tetanus in these age groups.

The results of the active tetanus immunization series of humans and guinea pigs suggest that the observed age and sex-dependent differences as well as the counterbalancing effects of LPS should be taken into account in future immunization protocols.

Liposome entrapped viruses (LEV) may serve as a complex gene-therapeutic delivery system by unifying the advantages of liposomal and viral vectors.

The principle of optimal adjuvation may offer a useful tool for the improvement of the efficacy of these therapeutical strategies.

4. CHAPPEL et al were not able to detect FasL on HMC cell lines, thus questioned the original hypothesis of Fas counterattack (Cancer Res ‘99;59:59). Our results with HMC cell lines supported the original hypothesis of Fas-counterattack. According to our observations, FasL expression on HMCs may decrease/cease gradually upon in vitro cultivation. This may explain the confusing results of CHAPPEL et al. Recent publications also support the original theory: *O'Connell: Ann N Y Acad Sci 2000 910:178*

The Fas-counterattack mechanism may serve as a new strategy of therapeutical tolerance-induction.

Fas-FasL autocrine loop of HMCs may be similar to Fas-related TNF loop of neuroblastomas (GOILLOT, Cancer Res ’92;52: 3194). Blocking Bcl-2 may destroy Fas-FasL autocrin loop, making HMCs defenceless against the attack of cytotoxic T lymphocytes. SINKOVICS J et al (Tampa, FL) has planned to deal with his subject further.

5. The analyzed features of SMPD1 gene (few and small introns, Alu 1 repeat element, GC-rich regulatory region, alternative splicing) are characteristic to imprinted genes (Nat Genet;12:234,’96). Based on these data in can be hypothesized that ASM gene (SMPD1) can suppress/counterbalance the anti-apoptotic effects of BWS-related growth-promoters, like IGF-II, among normal circumstances. Recent literary data are supporting this view. (Cell 86:189, '96; J. Exp. Med 187:897,'98; Biochem. Biophys. Res. Commun. 258:506 '99 ; FEBS Letters 452: 100, '99)

Our clinical and experimental data suggest that SMPD1, most likely at 11p15.4, is an imprinted, maternally expressed, BWS-and apoptosis-related growth suppressor gene. These

conclusions are in accordance with the "cluster-model of imprinting": Trends Genet 13: 330 '97 (i) as well as with the conflict theory of imprinting: Trends Genet 7: 45'91(ii).

Genomic imprinting may serve as a basic regulatory system of gene expression, thus, its therapeutical control may open a new prospect of gene therapy, immune therapy and cancer–treatment.

The exact localisation of SMPD1 to 11p15.4 as well as the construction and publication of the most comprehensive map of human chromosome 11 may add further details to the understanding of genetic regulatory mechanisms as well as the whole human genome.

Collaborating institutions - in alphabetic order (number of the topics in brackets)

All Children's Hospital of the University of South Florida, Tampa-St Petersburg, FL (Topic 4)
County Hospital , Győr, Hungary, (Topic 1)
Human Institute for Serobacteriological Production and Research, Budapest,: (Topic 3)
Independent Research Group for Genetics and Immunology, Budapest (Topics 3, 4, 5)
Institute for Enzymology of the Szeged Biological Centre of the Hung. Acad. Sci.,Budapest: (Topic 2)
Internatl.11 Chromosome Workshop, University of Nice, Medical Faculty, Nice, France. (Topic 5)
National Institute for Immunology and Haematology, Budapest: (Topic 2)
Phylaxia State Veterinary Institute, Budapest (Topic 3)
Semmelweis University Med. School, 2nd Dept. of Pediatrics, Budapest (Topics 1, 2, 5)
St. Joseph's Cancer Center Tampa FL. (Topic 4)
University (Med. School) of Pécs, Hungary: Inst. f. Microbiology an Immunology (Topic 3)
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