Session 1: Cancer

Monday 23 April 2001, Moderator: Dr. Zdenka Jonak

[09.40–10.00]

Identification of the antigen that is recognized by human monoclonal antibody CLN-IgG

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Purpose: CLN-IgG is a human monoclonal antibody that is secreted by a hybridoma that was created by a lymphocyte of a patient with cervical cancer and human B cell lymphoblast UC729-6. We identified and analyzed the antigen that is recognized by CLN-IgG.

Methods and results: (1) We purified the protein from the fraction of human glioma cell U-251MG by use of CLN-IgG coupled affinity chromatography. The protein that was isolated by SDS-PAGE was digested with Lys-C. The amino acid sequence of the peptides obtained was determined by protein sequencer. (2) We carried out 2-dimensional electrophoresis of cytoplasmic and membrane proteins. The spot that showed affinity to CLN-IgG was fragmented with trypsin, and the peptide obtained was analyzed by mass spectroscopy. Experiments (1) and (2), respectively suggested that the antigen that is recognized by CLN-IgG is vimentin. Therefore we cloned the vimentin gene and carried out RT-PCR using the total RNA of U-251MG as template in order to determine the epitope of CLN-IgG. Moreover we prepared GST fusion protein of all domains (head, coil 1, coil 2, tail) of vimentin, and studied its affinity with CLN-IgG. As a result we found that CLN-IgG only bound to fusion protein that contained the coil2 domain.

Conclusions: The antigen that is recognized by CLN-IgG is vimentin, and the epitope is located on the coil2 domain.

Keywords: Human monoclonal antibody, vimentin.

[10.00–10.20]

lmmunotherapy for stomach carcinoma with the human monoclonal antibody SC-1 and the new DAF apoptosis pathway

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Human cancer patients are the best source for tumor-specific and tumor-reactive reagents (cells, factors, antibodies) and the human hybridoma technology offers the only and unique technique, to get both in one step: identification of new targets on tumor cells with new tumor-cell related mechanisms and complete human antibodies for diagnostic and therapeutical purposes. We have recently described the human monoclonal antibody SC-1, which was isolated from a patient with gastric cancer. The antibody binds to 25% of tested intestinal-type and 70% of diffuse-type stomach adenocarcinoma. The moderately affinity-maturated 1gM antibody (DP49) induces specific apoptosis in vitro and in vivo in animal studies. Used in a clinical trial with 44 stomach carcinoma patients, significant apoptotic and regressive effects on tumor cell proliferation in primary tumors and metastases could be observed. The human antibody SC-1 binds to a new modified form of membrane-bound CDSS (DAF-B, decay-accelerating factor), that is specifically overexpressed on stomach carcinoma cells and absent on other tumor cells or healthy tissue. DAF-B therefor exists in two different glycosylated forms on stomach carcinoma cells, in addition to the ubiquitously distributed 70 kD isoform, which protects cells from lysis through autologous complement, a specific modified 82 kD DAF-S is coexpressed. Crosslinking of the GPI-finked molecule DAF-BISC-1 isoform by antibody SC-1 results in its upregulation, followed by its internalization and disappearance from the membrane. The DAF-wild-type on the other hand is slightly increased and shows then a stable expression. A tyrosine phosphorylation of 60, 75 and 100 kD proteins and a serine dephosphorylation of a 35 kD protein is observed shortly after SC-1 induced

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apoptosis. The 35 kD protein was identified to be hnRNP Al, which is cleaved into different products after the induction of apoptosis. The dephosphorylation at the serine residue is important for apoptosis, since the amount of apoptotic cells can be decreased by the specific serine/threonin phosphatase inhibitor okadaic acid and serine kinase inhibitor H7 leads to a dose dependent increase in apoptosis. Cleavage of hnRNPI Al involves caspase 2 and cytokeratin 18 is processed by caspase 6. Other investigated caspases like caspases 1, 3, and 9 seem not to be involved in this process, because inhibition of these enzymes results in an increase of apoptotic events. Stomach carcinoma belongs to most dangerous malignant diseases worldwide. Treatment is mostly limited to radical gastrectomy, lymphadenomectomy and in cases of irresectable tumors to chemotherapeutical approaches. But even then, according to the number of people killed worldwide by this cancer, the prognosis is very poor and there is a big need for additional adjuvant therapy. Human antibodies like SC-1 give hope for more effective and less harmful treatment of carcinoma and for the understanding of tumor-related mechanisms.

[10.20–10.40]

Human anti-idiotypic antibodies against therapeutic anti-GD_2-antibody ch14.18 cloned by phage display from a neuroblastoma patient as a possible tumor vaccine

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Neuroblastoma is a tumor of neuroectodermal origin with a poor prognosis in advanced stages of disease. In clinical trials using chimeric monoclonal antibody ch14.18 directed against GD2, a ganglioside overexpressed on neuroblastoma cells, objective responses including complete remissions could be seen. In the course of the clinical trials with ch14.18 (Ab1) we observed that patients producing anti-variable region antibodies, so called anti-idiotypes (Ab2), against ch14.18 showed a more favorable prognosis than the ones with little immune response. According to Jerne's network theory a subset of these anti-idiotypic antibodies form an 'internal image' of the antigen and thus are able to induce antibodies (Ab3) directed against the original antigen. These anti-idiotypic antibodies can function as an internal tumor vaccine. To further investigate these anti-idiotypic antibody responses we chose a neuroblastoma patient who showed high titers against the therapeutically given antibody ch14.18 as well as against 14G2a, the original mouse antibody which only shares the variable region with ch14.18. His serum also was capable of inhibiting the binding of ch14.18 to GD2, a necessary condition for internal image antibodies. To clone these anti-idiotypes, we constructed antibody phage display libraries using the patient's B-cells as a source. After repeated biopanning on 14G2a we selected several clones that bound highly specific to 14G2a as well as ch14.18. Two of the clones analyzed further could inhibit the binding of ch14.18 to GD2, just as the serum did. Currently we test the clones in vivo if they are capable of inducing Ab3 and therefore may be useful as a tumor vaccine against neuroblastoma.

[11.20–11.45]

Use of monoclonal antibody to detect a 90 kD tumor antigen-specific immune complex in sera of cancer patients

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Immune complexes (IC) have been observed in circulation of cancer patients; however, their application for clinical evaluation of the disease has been hampered due to antigen non-specific nature of the IC detection assays. This investigation was undertaken to develop an antigen specific-IC detection assay using a murine monoclonal antibody. An autoimmunogenic 90 kD glycoprotein tumor antigen (TA-90) was detected in human solid tumors. After purification, this antigen was used to develop a murine monoclonal antibody (MoAb) of IgM isotype. Blocking studies revealed that the epitope on TA-90 recognized by the MoAb was different from those recognized by human autoantibodies. Also, the MoAb did not react with human serum proteins. The MoAb was immobilized on a solid matrix and used to capture the TA-90 specific IC present in the test serum. Capturing of the antigen-specific IC was assessed using goat anti-human IgG conjugated to alkaline phosphatase. An ELISA value of > 0.410 OD_{405} (mean plus 3 SD of normal) was considered positive. The incidence of the TA-90 specific IC in sera from 419 cancer patients with various types of solid tumors was significantly higher ($p < 0.05$) than in sera from 250 self-proclaimed normal individuals (55.9% vs 3.2%). The age and gender of the serum donors did not affect the incidence of the reactivity. To determine clinical

utility of the MoAb based TA-90 specific IC retroactively collected sequential serum samples from 192 melanoma patients (105 patients with recurrence within 10 years and 87 patients with no recurrence for more than 10 years of follow-up) were analyzed. Results revealed that 69% (72/105) patients in the recurrent group were positive for TA-90-IC compared to 23% (20/87) in the non-recurrent group ($p < 0.05$). Analysis of the data by the Log-rank statistical method revealed that the two groups differed significantly in terms of their recurrence rates. We conclude that the MoAb based TA-90-IC assay represents a new approach to tumor marker detection that can be used to assess prognosis of cancer patients after surgical resection of the tumor.

[11.45–12.05]

The development of novel immunotoxins for treatment human melanoma

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Four new immunotoxins on base anti-HMW-MAA Mabs and ricin and viscumin have been prepared (763.74/RTA, 763.74/MLA, 225.28/RTA, 225.28/MLA). Binding of the IT with different melanoma cell lines has been investigated by immunofluorescent methods. Melanoma cell lines Melur, Colo 38, F-01, FM-3 were chosen as the main target cells for cytotoxic tests. Cytotoxic activity of each IT was compared with that of the free antibodies, the free A-chain, and the mixture between the antibody and the A-chain. The most specific and active IT was 763.74/MLA. LD50 of this IT was 1,2*10-10M. This fact is very important because only Its that have LD50 of 10(-10M) or less are considered suitable for clinical use. Different ways to increase cytotoxic activity of IT were used (involving combinations of different Its, secondary antibodies against mouse IgG, and the purification on the affinity column HiTrap with immobilized anti-id mABs). Only NH4CL had an inconsiderable effect on 763.74 IT activity. We also investigated the ability of ITs to induce cell death via apoptosis. The activity of ITs and native ricin and viscumin to induce apoptosis was compared. Melanoma cells FM3 were cultured in the presence of cytotoxic agents causing 90% cell death. After 24 hours of cultivation apoptotic cell death caused by 225.28/MLA was 60%, by 225.28/RTA 17%, by viscumin 100%, and by ricin 70%. To investigate intracellular transport, we obtained mABs against different epitopes of A- and B-chains of viscumin. Eighty linear antigenically active sites were revealed in Mistletoe lectin A-chain viscumin by octapeptide scanning. Using the hybridoma produced mABs against MLA, we showed that during intracellular transport of viscumin its subunits dissociated and MLA unfolded before translocation. These are very important results because the process of intracellular transport of plant toxin is poorly understood (especially the process of translocation). It is also of practical use. We plan to optimize IT intracellular transport by including the translocation domain in gene-engineering construction of IT. The renaturation rate of MLA and RTA, detected by mABs TA71, TA75, TA77, was different. These differences may influence the cytotoxic activity of IT based on MLA and RTA. Currently, we are dealing with a chimeric recombinant construction based on MLA and dyphteria toxin translocation domain. The results obtained will help us develop recombinant IT which can be used for treatment melanoma patients.

[12.05–12.25]

Gene gun-mediated DNA vaccination with idiotype /granulocyte-macrophage colony-stimulating factor fusion gene induces antibody responses against the tumour-associated antigen, EpCAM in EpCAM-transgenic mice

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Vaccination studies using anti-idiotypic antibodies (ab_2) as surrogate tumour-associated antigens have been reported and shown the ability to induce immunity against tumours in several animal models and clinical trials. However, the optimal design of an ab_2 vaccine remains unclear. In this study a human ab_2, SM262, was used to determine the modulatory effect of two design features on humoral response: the presence of a xenogeneic vs syngeneic F_c domain and fusion to granulocyte-macrophage colony-stimulating factor (GM-CSF). SM262 was isolated from a patient sample as a result of treatment with the murine monoclonal antibody, Panorex (ab_1), which recognises the human epithelial cell adhesion molecule, EpCAM. Plasmid DNA constructs encoding the variable regions ($v_H + v_L$) of SM262 or an irrelevant antibody with either mouse or

human F_c portions, both with and without GM-CSF fusions, were delivered by gene-gun into C57BL/6 mice (1 μg DNA/dose on day 0 and 28). In addition, to address whether immune tolerance to EpCAM can be broken by vaccination with these constructs, responses in C57BL/6 mice expressing the transgene for human EpCAM were also evaluated. All constructs induced humoral responses that recognised SM262 (anti-anti-idiotypic antibodies, ab_3) in both wild type and transgenic mice. The highest ab_3 titers were elicited when constructs with both xeno-F_c and GM-CSF fusion were used. Human F_c portion without GM-CSF fusion as well as mouse F_c with GM-CSF fusion induced higher ab_3 titers as compared to mouse Fc without GM-CSF. The majority of the ab_3 responses were generated against the CDR region of SM262 when syngeneic-F_c was used. Xeno-Fc induced similar levels of anti-CDR and anti-allotypic antibodies. The SM262 plasmids induced ab_1 like antibodies (ab_1'), that were able to inhibit Panorex-SM262 interaction and recognised the nominal antigen, EpCAM, in both wild type and transgenic mice. However, higher ab_1' titers were induced when SM262 was fused to GM-CSF. Xeno-F_c alone did not have a modulatory effect on induction of ab_1'. Pre-immune and control sera were negative for ab_1'. There was no correlation between ab_3 and ab_1' titers. Our data suggests that tolerance to EpCAM can be broken by gene gun-mediated ab_2 DNA immunisation and fusing GM-CSF to ab_2 can increase the magnitude of responses elicited. These results might have important implications for the design of clinically relevant gene gun vaccination strategies for tumour immunotherapy.

[12.25–12.55]

Phase I/II trials of two genetically engineered antibodies SGN-10 (BR96 SFv-PE40) and SGN-1S (ChBR96-Dox): A pharmacokinetics and immunogenicity comparison

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Abstract not received.

[14.00–14.30]

Immunogenetic analysis reveals that epitope shifting occurs during B-cell affinity maturation in primary biliary cirrhosis

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Primary biliary cirrhosis (PBC) is a liver disease characterized by serum autoantibodies against the pyruvate dehydrogenase complex (PDC) located in the inner mitochondrial membrane. The predominant target in PDC has previously been localized to the inner lipoyl domain (ILD) of the E2 subunit. The etiology of PBC is unknown, although molecular mimicry with bacterial PDC has been proposed. In this study, we have investigated the development of the autoimmune response by analyzing the structure of a human monoclonal autoantibody derived from a patient with PBC. The MoAb had the typical disease-associated specificity for the E2 subunit, recognizing a determinant in the ILD. To determine the clonal history of the MoAb, the Fab region was expressed in phage, somatic mutations in the V-regions were selectively removed, and the effect on binding to autoantigen assessed. Reversion of the V_H sequence to germline sequence changed the specificity from the site in the ILD to a different epitope within E2. Reversion of both V_H and V_L to germline sequence abolished reactivity with all sites in E2. No reactivity to bacterial PDC was detected with original or modified Fabs. We hypothesize that the IgM on the surface of the naive B-cell first recognized an as yet unidentified antigen, and that accumulation of somatic mutations results in an intermolecular epitope shift towards a different epitope within E2. Further mutations result in the specificity being redirected to the ILD. The fact that the naïve B cell is not triggered by the autoantigen, but that it apparently acquires graded autoantigenic specificity during affinity maturation, has implications for our understanding of the development of autoimmune disease.

[14.30–14.50]

Efficient and rapid clone selection of human-antibody producing xenohybridomas by flow cytometry and cell sorting according to single cell specific production rate

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A method that allows flow cytometric sorting of single live cells according to their protein secretion rate has been used to improve the procedure of human hybridoma subcloning and to help especially in the selection of rare cells with high production rates. Previous work has shown that by using this method the work load required to obtain a high producing subclone can be reduced by a factor of ten, while the specific production rate of the resulting clones will be even higher than that obtained by traditional limited dilution cloning. A human-mouse xenohybridoma cell line

producing a human antibody against HIV-1 was used to optimise and evaluate the technique. To allow the analysis of the amount of secreted antibody on single cells, the cell surface is covered with an affinity matrix consisting of botinylated surface proteins, an avidin bridge and a biotinylated antibody against the secreted human antibody. After incubation and binding of the secreted antibodies to the affinity matrix on the cell surface, cells are stained with a second, fluorescent-labelled antibody against the product. It was necessary to optimise the concentrations of all components (biotinylation reagent, avidin and "catcher" antibody) for each hybridoma cell line. The kinetics of antibody secretion as well as data on the correlation between ELISA-obtained specifc production rates and secretion signals measured by flow cytometry are presented. Optimisation of the coating procedure, especially of the concentration of the biotinylation reagent NHS-succinimidyl-biotin, resulted in improved cloning efficiency, which enhanced sorting output. An unstable subclone was used to demonstrate the usefulness of the technique. This subclone showed a decrease in specific production rates from initially 10 pg.cell^{-1}.day-1 to 0.2 pg.cell^{-1}.day^{-1} within 8 weeks. Cells were sorted for high production rates after 5 weeks (when specific production rate was 2 pg.cell^{-1}.day^{-1}) and after 8 weeks. The resulting clones both had a specific production rate of 6 pg.cell^{-1}.day^{-1}, which was stable for 25 weeks and 15 weeks, respectively. Thus one sorting step into one microtiter plate resulted in the establishment of clones with significantly improved stability, while requiring a minimum of expenditure. Further experiments will focus on the inclusion of additional sorting criteria like antibody specificity and the selection of peripheral B-lymphocytes. Sorting of antigen specific hybridomas directly after fusion would possibly enable their selection even in unprimed samples. Due to the reduced work load required the selection of cells with rare properties becomes feasible.

[14.50–15.10]

Human B cell growth and differentiation in the spleen of immunodeficient mice

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Human monoclonal antibodies (HuMAbs) have therapeutic potential against infectious diseases and cancer. Their production has been hampered by ethical constraints preventing the isolation of antigen-specific activated B cells by in vivo immunisation. Alternatively, severe combined immune deficient (SCID) mice, transplanted intraperitoneally with human peripheral blood leukocytes (Hu-PBL), allow the in vivo stimulation of human Ab responses without the usual constraints. Unfortunately, human B cells only represent a minor fraction of the surviving graft, they are scattered all over the animal body, and are thus hard to isolate for subsequent immortalisation procedures. To prevent this dispersion and to provide the human B cells with a niche for expansion and maturation, SCID mice were engrafted with Hu-PBL directly into the spleen. Simultaneously endogenous murine natural killer (NK) cell activity was depleted by treatment with an anti-mouse interleukin-2 (IL-2) receptor beta chain antibody. During engraftment, human B lymphocytes became activated, divided intensely, and differentiated into plasmacytoid cells. In vivo exposure to a recall antigen after cell transfer induced expansion of antigen-specific B-cell clones. One week after inoculation, human B cells were abundant in the spleen and could easily be recovered for fusion with a heteromyeloma line. This resulted in the formation of stable hybridoma cell lines that secreted antigen-specific HuMAbs. Transplantation of human lymphoid cells in the spleens of immune deficient mice thus represents a model for the study of human, T cell dependent B-cell activation and proves to be an excellent tool for the successful production of HuMAbs.

[15.10–15.40]

Integrating XenoMouse^{TM} technology and genomics to create new therapeutic human monoclonal antibodies

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Genomic and proteomic initiatives have identified hundreds of novel genes and gene products as targets against which antibody-based therapeutics for diseases such as cancer, inflammation and autoimmunity may be developed. Now, the challenge is to create a facile, relatively high throughput process for target validation that also can lead to candidate therapeutic antibodies. Abgenix's XenoMouse technology enables the generation of a broad panel of high affinity, fully human monoclonal antibodies against multiple epitopes. The

XenoMouse mice are genetically engineered to contain stable megabase-sized yeast artificial chromosome transgenes on a genetic background of functionally inactivated mouse IgH and Igκ loci. The human IgH and Igκ transgenes contain the majority of the human V_H and V_κ gene repertoire, Cμ and Cδ, in germline configuration as well as either C$_\gamma$1, C$_\gamma$2 or C$_\gamma$4. These transgenes efficiently support both the development of B cells and, after immunization, the generation of robust and diverse fully-human primary and secondary immune responses in the XenoMouse strains. Fully human monoclonal antibodies from XenoMouse mice are in Phase I and Phase II clinical trials for cancer and psoriasis. Abgenix's subsidiary, ImmGenics, has developed the SLAM technology, a rapid and efficient method that can obviate the need for hybridoma generation. In the SLAM process, the entire fully human immune repertoire of XenoMouse animals can be assayed for antigen-specific antibodies. When combined with high-throughput screening assays, the SLAM process allows the recovery of unique antibodies based on criteria such as high affinity, high potency or rare activities. Combining XenoMouse technology with SLAM technology allows the rapid functional analysis of targets from genomic and proteomic initiatives and the subsequent recovery of fully human monoclonal antibodies of potential therapeutic value.

[16.10–16.35]

Anti-GBM disease in XenoMouse$^{\mathrm{TM}}$ mediated by human antibodies versus α3(IV)NC1 collagen

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To establish an anti-GBM disease model more relevant to the human form of the disease, XenoMouse II, that produce human IgG2γd2 2κ) constitutively, were immunized with various forms of α3(IV)NC1 GBM collagen, including (i) bovine NCI collagen, (ii) baculovirus expressed recombinant α3(IV)NCI, and (iii) *E. coli* expressed rα3(IV)NC1 preparations. All mice developed high titer, human anti-GBM antibodies (ELISA) and proteinuria (2.5–10.7$\times$control). Light microscopy showed proliferative glomerulonephritis with (i) 40%, (ii) 57%, and (iii) 31% crescents respectively. By direct immunoflourescence all mice had linear IgG deposits along the murine GBM and TBM with weaker staining for C3. Fully human monoclonal anti α3(IV) Ab (Ig2κ) have been isolated, and some produce nephritis after transfer to XenoMouse II. In particular, MAbF1.1, derived from an animal immunized with native bovine NC1 collagen bound to native bovine dimers as well as to 293 fetal kidney cell, and E. coli expressed recombinant human *f*Á3(IV)NC1 by ELISA and Western blotting. By indirect IF, MAbF.1.1 produced patchy linear GBM staining of fixed and unfixed normal human glomeruli in a manner similar to serum antibodies from diseased patients. MAbF1.1 is encoded by VH 4 and VL κ3 gene families, and comparison to other monoclonal reagents is ongoing. Normal SJL mice given MAbF1.1 develop linear basement membrane deposits of human IgG associated with mild glomerulonephritis and proteinuria (1.98 mg Upr/24 h vs. 0.72 mg/24 h, control); the nephritis is accentuated by administration of CFA prior to MAbF1.1 ($>$ proliferation, 4.5 mg Upr/24 h). Administration of MAbF1.1 to XMII results in linear IgG deposits, crescentic glomerulonephritis and heavy proteinuria (10.6 mg, Upr/24 h). The results indicate that anti-GBM disease can be initiated in susceptible strains by human anti-α3(IV)NC1 antibody with an single specificity for α3(IV)NC1. This model should be useful to test therapeutic strategies targeted at human anti-α3(IV)NC1 autoantibodies and the B cells that produce them.

[16.35–16.55]

Preparing a human IgA for cancer immunotherapy

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Abstract not received.

[16.55–17.15]

Main characteristics of the immunoglobuline repertoire of B lymphocytes infiltrating human breast medullary carcinoma

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Introduction: The presence of immunocompetent cells in solid tumors may reflect ongoing immune responses against transformed cells that contribute to the spontaneous tumor regressions. The tumorspecific reactivity of T lymphocytes infiltrating solid tumors (TIL-T) are highly investigated in terms of T cell receptor usage, specific T cell clones recognizeing peptides pre-

sented by various tumors. Our objective is to confirm the tumor specific reactivity of tumor infiltrating B lymphocytes (TIL-B). There is very poor if any information in terms of TIL-B cells and the immunoglobulin variable (Ig V) region repertoire has not been revealed at all.

Study design: To answer this question about 200 expressed TIL-B immunoglobulin heavy and light chain variable regions were cloned and comparatively analysed at nucleotide sequence level from medullary breast carcinoma (MBC) where massive B and plasma cell infiltration correlates with favourable prognosis. By comparison to the EMBL database using BLASTn search engine the closest expressed and by IMGT the closest germline sequences were defined.A model systhem was set up in order to reveal the potential tumor antigen recognizing capacity of B lymphocytes infiltrating solid tumors.

Results and conclusions: The TIL-B Ig VH and VL sequences could be classified into clusters, families and subgroups, based on their very high homology level or identity. We found some clusters (composed of overexpressed sequences), that showed very high homology (98.5%) through the whole Ig V DNA sequence (including CDR3 region as well) of clones with a proven specific binding capacity to tumor cells (e.g.: disialogangliosides on neuroblastoma). The TIL-B clones of interest are to be selected and expressed in order to test for specificity first against gangliosides and glycosphingolipids on breast cancer cell lines, as provement of the theory. Based on our new findings the potential tumor antigen binding capacity of TIL-B can be suggested.