SESSION 2 : INFECTIOUS DISEASES

Human hyperimmune IgG from immunized plasma donors for treatment and prevention of infection with multi-drug resistant *Staphylococcus aureus*

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The emergence and rapid spread in hospitals of multi-drug resistant bacterial infections such as vancomycin resistant enterococci, methicillin resistant staphylococci and penicillin resistant pneumococci, have drawn attention to the need for alternative therapies, including immunotherapies, to combat these. Capsular polysaccharide (CP) vaccines were developed and proved to be effective in preventing some bacterial infections including pneumococci and meningococci. However, active immunization of several at-risk populations is impractical. Active immunization requires both immuno competance and a window of time, from a few days to a few weeks, for the body to mount an appropriate immune response. Groups such as trauma patients, low birthweight neonates, patients on chemotherapy, and many surgical patients are at high risk for infection and either have compromised immune systems, or do not have enough time to mount an effective immune response to active immunization before the onset of infection. For these indiciduals, passive immunization in the form of a vaccine-stimulated hyperimmune globulin may be a practical means of preventing or treating infections. The clinical use of human polyclonal (IgG) preparation is well established and immunoglobulins are widely used in hospitalized patients. We immunized volunteer plasma donors with a *S. aureus* CP conjugate vaccine, collected plasma, and fractionated it to produce a hyperimmune IgG. These stimulated immunoglobulin (SIG) preparations were compared to standard commercial intravenous immunoglobulin (IVIG). The specific antibody titer to *S. aureus* CP in SIG was >40-fold higher than that in IVIG. In addition, SIG preparations from immunized donors had >5-fold higher affinity to the CP antigens than did the antibodies in the standard IVIG. Moreover, the SIG antibodies had a higher efficiency in mediating the deposition of complement, as measured by C3b deposition, than did standard IVIG. SIG and standard SIG preparations were tested in animal models for *S. aureus* infections. Either intraperitoneal or subcutaneous administration of SIG preparations provided significant protection over standard IVIG against bacteremia, organ (kidney and liver) seeding, and lethality in animals challenged with *S. aureus*. These data suggest that specific SIG antibodies are likely to be more effective in the treatment and prevention of staphyloccal infections than standard IVIG. Since the use of human polyclonal antibodies has long been shown to be safe, our data suggest the feasibility of using vaccine-stimulated IgG to develop human hyperimmune intravenous immunoglobulins which target specific infections. This approach may prove to be useful in combating emerging antibiotic resistant bacterial pathogens.

Monoclonal and single-chain antibodies against different diagnoses and cell surface antigens: immunological tools for the structural and functional characterization of bacterial isolates

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Monoclonal antibodies are specific and highly sensitive tools for the rapid identification and quantification of bacteria in complex ecosystems. Advances in the fields of antibody engineering contributes to improvement of these immunological tools and their application in different detection systems. In this study we compared the conventional hybridoma technique with the selection of antibody fragments derived from a synthetic library with regard to establishing a screening methodology for specific bacteria and bacterial proteins in ELISA, dot blot, colony blot and immunofluorescence. Several MAbs were raised against different dioxygenases, which are intracellullar enzymes and play an important role in biodegradation of PCBs. The immunological tools were highly specific for the three isoenzymes bphC1, bphC2, and bphC3 of the 2,3-dihydroxybiphenyl 1,2-dioxygenase (2,3-DHBD) from *Rhodococcus globerulus* P6, the 2,3-DHBD from *Burkolderia cepacia* LB400 and the 2,2',3-trihydroxybiphenyl 1,2-dioxygenase from *Sphingomonas* sp. RW1. All immunological probes were functional in ELISA, dot blot and colony blot. Immunofluorescence studies with isoenzyme overexpressing *E. coli*s were successful and therefore it should be possible to develop an immunohistochemical approach for the identification of dioxygenase-expressing bacteria on the single cell level. The detection and quantification of the enzymes in the gram-positive parental strain *R. globerulus* P6 is in progres. As an alternative system scFv were derived from a synthetic library against cell surface antigens of *Sphingomonas* sp. RW1. These antibodies are able to detect the bacteria in different immunological assays with the same sensitivity and specificity as the conventional MAbs. In a next step, recombinant antibodies against the different dioxygenases will be selected and used as functional probes. These small molecules (scFvs) which have a molecular weight of ~ 30 kDa seem to be promising in penetrating even gram-positive cells and detecting the enzymes intracellularly. Further studies are in progress to use the monoclonal and recombinant antibodies in environmental samples as functional and structural probes, allowing the screening of samples and monitoring bacteria *in situ*.

Generation of biologically active anti *Cryptococcus neoformans* isotype switch variant MAbs

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Cryptococcus neoformans causes life threatening meningoencephalitis in approx. 10% of patients with AIDs. In the setting of severe immunosuppression, cryptococcal infections are usually incurable and often fatal. The difficulties encountered in treating these patients have renewed interest in passive antibody administration as an adjunct to antifungal therapy. Passive administration of MAbs to *C. neoformans* can alter the course of infection in mouse models. However, the effectiveness of these antibodies appears to depend on isotype and specificity. 3E5.γ3 is an IgG3 secreting MAb to *C. neoformans* capsular glucuronoxylomannan. The antibodies do not prolong survival or reduce organ fungal burden. In contrast, the IgG1 switch variant of 3E5 significantly prolonged survival of infected animals. Comparison of isotype protection efficacy requires families of MAbs with identical fine specificity and different constant region. The generation of such families of hybridomas is not always possible either because of the immune response which allows the production of limited immunoglobulin classes or subclasses or because of the low frequency of isotype switching of such hybridomas *in vitro*. Using acridine orange and an ELISA spot assay we have been able to identify and isolate the entire set of isotype switch variants: γ1, γ2b, γ2a, ε, α. All antibodies bind glucuronoxylomannan and share identical binding site specificity as determined by anti-idiotypic MAb. The functionality of these antibodies was demonstrated by their ability to enhance phagocytosis and anti-fungal efficacy of human THP-1 macrophage cells. The role of Ig subclass in conferring protection to *C. neoformans* will be discussed.

Human monoclonal antibodies to viral peptides

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B lymphocytes bearing immunoglobulin receptors specific to V3-loop of gp120 of HIV-1 and to EBNA-1 antigen of Epstein-Barr virus (EBV) were isolated from freshly removed human tonsils. Positive selection of the antigen-specific B cells was done using tosyl-activated Dynabeads sensitized either by synthetic peptide MN-24 corresponding to 302-322 residues of gp120 HIV-1, or by peptide p107 of EBNA-1. The target cells with 3-5 attached magnetic beads (rosettes) were separated from non-rosetting cells using magnetic device and transformed by EBV. After transformation cells were cultivated in 96-well flat bottomed plates (100 cells/well) on the irradiated human embryonic lung fibroblasts in complete RPMI 1640 OptiMEM medium supplemented with 10% of fetal calf serum. Human immunoglobulin (Ig) and antibodies (Ab) to peptides were determined by ELISA. The first grown clones became visible after one week of cultivation. After 5 weeks of cultivation cells from Ab-positive wells were transferred to 24-well plates. One of the Ab-positive culture was expanded. This culture continues to produce IgM Ab to p107 over two years. Transformed cells are used for receiving human/mouse and human/human hybridomas.

Neutralization of RNA-phage infectivity by anti-fr MAb and CDR-peptides that determine its specificity

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RNA-bacteriophages are a highly conserved group of the simplest single-stranded viruses of defined structure. In spite of this, less is known about the antigenic and immunogenic properties of the phage particles. A knowledge of spatial structure of the bacteriophage fr as well as the very simple arrangement of phage neutralization test stimulates our interest to understand molecular details of the nature of virus neutralization and antibody-antigen recognition process. This communication reports on the construction and characterization of a mouse hybridoma FR52 secreting neutralizing MAb specific for RNA-phages fr, MS2 and GA. Using the cDNA-PCR technique, we have cloned and then sequenced the genes encoding the variable domains of the MAb FR52 heavy and light chains. The CDR principles were chemically synthesized and were tested for their ability to neutralize the activity of RNA-phages fr, MS2 and GA. The CDR-derived peptides H2, L2 and L3 interacted with the fr phage particles and neutralized fr phage activity. Two of these peptides - H2 and L3 - also had the ability to neutralize partly the activity of related bacteriophage MS2, but L1 and especially L3 neutralize the activity of the RNA-phage GA. These results raise the possibility that simple CDR-peptides may serve as a new class of antiviral molecules.

Generation of human MAbs against respiratory syncytia virus

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Respiratory syncytial virus (RSV) can cause severe lower respiratory disease in infants and the elderly. Immunity is incomplete and reinfections are common at all ages. Passive immunotherapy in infants has shown success with high doses of human poly-IgG and therefore has encouraged efforts to develop human MAbs. Key hurdles in their development, as potential agents of therapeutic utility are the supply of B-cells activated to produce antibodies against the antigen of interest, the instability of human hybridomas, and the difficulty in obtaining human antibodies against self antigens. Several new technologies are overcoming these issues. Here we present our application of two such technologies - hybridoma reuse and antibody combinatorial cloning - to the isolation of human MAbs directed against RSV. These two technologies both utilize the same starting source, i.e. human B-lymphocytes obtained from tissue samples such as spleen, peripheral blood lymphocytes (PBLs) or bone marrow. They also share a common endpoint: the expression of candidate MAbs in surrogate cell lines (COS, CHO). The technologies differ fundamentally in the method of capturing MAbs with the desired functional property of neutralization of RSV. For combinatorial cloning, the heavy and light chains were cloned as populations by PCR and then randomly associated for display as a 'combinatorial library' of Fabs on the surface of bacteriophage. Following phage selection against recombinant RSV F envelope antigen, Fabs were recovered and screened for their antiviral activity in *in vitro* assays. By this approach, we identified a dominant non-neutralizing response common to three different individuals. However, blockade of this epitope during a repeat of the phage selection process yielded high affinity Fabs recognizing distinct epitopes. The advantage of this approach was the apparent effective capture of the immune response and the antigen directed affinity selection. Hybridoma rescue was an adaptation of conventional technology whereby the B-cells were first stabilized by cell fusion and/or viral transfection, and then screened for function prior to rescue of individual heavy and light chain genes by PCR cloning using family specific heavy and light chain primers. This approach should yield MAbs that reflect natural pairings of heavy and light chains *in vivo*. Two issues thwarted this approach. The level of antibody produced by the hybridoma cultures was rarely sufficient for bioassay, necessitating selection based on binding properties. Secondly, the success of direct PCR rescue was often limited by the polyclonal nature of the hybridomas, low cell numbers, or rapid loss of IgG genes. In a merger of the two technologies, we utilized 'mini-combinatorial' cloning/phage display of the binding positive hybridoma cultures to overcome the problem of polyclonality. Functional antibodies were rescued from the 'clone' of interest and screened for antiviral activity. Human MAbs generated by these technologies, their derivation, properties and interrelationships will be discussed.

Production of monoclonal antibody against *Neisseria meningitidis* that recognize specific and cross-reactive antigens

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Meningococcal meningitis and septicemia caused by *Neisseria meningitidis* continue to be worldwide public health problems. The mortality rate is high, and the disease occurs in epidemics. Beginning in 1988, the incidence of meningococcal disease in Greater Sao Paolo exceeded 4.06 per 100,000 suggesting a new epidemic in the region. This is different from previous epidemics, as it was caused by serogroups B and C. Meningococcal cell surface constitutes that part of the organism which interacts most intimately with the host and meningococcal cell surface structures are known to be critical in coloni zation, invasion and disease pathogenesis. Clearly, for a better understanding of the host immune response to *N. meningitidis*, more information about the characteristics of specific antigens of *N. meningitidis* is necessary. Hybridization technology has made it possible to use MAbs in immunological probes. Clone 8C7Br1 was obtained form a fusion of mouse spleen cells with the murine myeloma cell line X63Ag8.653. Mice were immunized with two doses of live meningococci given both intra-peritoneally and -venously three days before fusion. The meningococcal strain used in this study was B:4:P1.9 recovered from patients with meningococcal disease in Brazil. The MAb was initially based on their binding with homologous strain by ELISA. Immunoblotting of SDS-PAGE resolved *N. meningitidis, N. gonorrhoeae, H. influenzae* b, *E. coli, B. pertussis* and *B. subtillis* were used to demonstrate antibody cross-reactivity. The MAb 8C7Br1 recognized their target antigen 50 kDa in different serotypes of *N. meningitidis, N. lactamica, N. gonorrhoeae,B. pertussis* Meanwhile, with *H. influenzae, E. coli* and *B. subtillis* the monoclonal recognized a protein of 60, 65 and 70 kDa, respectively. The interesting finding that different electrophoretic mobilities were obtained when reacting MAb 8C7Br1 with Brazilian *N. meningitidis, H. influenae* b and *E. coli* is presently under investigation. We have generated the hybridoma cell line which produce IgM antibody isotype reactive against specific antigens of different serogroups and serotypes of *N. meningitidis.* These new MAbs will give more information about antigenic variability and can be used to screen a recombinant gene library. Sequence studies and epitope mapping will give us more information about the protein and its possible application as vaccine component.

Evidence for an antigen driven selection process in human autoAbs against acetylcholine receptor

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Autoantibodies to the nicotinic acetylcholine receptor (AChR) play a central role in the neurological symptoms associated with myasthenia gravis (MG). A better knowledge of the structural organization and the mechanisms leading to the production of these autoantibodies may help in understanding the pathogenesis of the disease. To achieve this, four IgG anti-AChR monoclonal autoantibodies obtained from previous work were derived from lymphoid cells of MG patients. Two of them (MH1 and MH6) were capable of modulating *in vitro* the expression of AChR at the surface of TE-671 cells. We report here the complete nucleotide sequence of the heavy and light chains of these four antibodies. Although it is difficult to address the issue of VH gene usage in anti-AChR autoantibodies because of the limited number of clones studied, our results associated with others which appeared in the literature point to non-stochastic usage by anti-AChR antibody of some defined VH genes belonging to VH2 and VH5 minifamilies overexpressed in the fetal repertoire.
The second and major aim of this work was to assess the role of an antigen driven selection process in the production of anti-AChR autoantibodies. When comparing the expressed sequences in their closest germline counterparts, it appeared that all four studied clones displayed numerous mutations in VH regions. In particular, MH1 and MH6, characterized by their AChR modulating capacity, displayed a higher than expected number of mutations and replacements occurring in CDR regions. These data point to an antigen driven selection process. On the contrary, the mutational process observed in the MH5 clone was borderline and that of MH7 was compatible with a random process. Interestingly, when comparing mutations in heavy and light chains, a significantly lower number of mutations were expressed in light chains for the four clones.

Production of anti-HBx/CD3 BSAB secreting hybrid-hybridomas and application of the BSAB retargeting effector cells for lysis of human HCC xenografts in nude mice

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Evidence indicated that the *x* gene of human HBV can cause cancer in transgenic mice; moreover, HBxAg was so far the most frequent and strong antigen among those HBV markers expressed in hepatocellular carcinoma (HCC) tissues. Aimed to enhance killing of HCC by effector cells, we established an anti-HBx/anti-CD3 hybrid-hybridoma by fusion of anti-HBx hybridoma cells with FITC-labelled anti-CD3 HAT sensitive cells, and followed by FACStar sterile cell sorting, HAT selection and eventually verified by ELISA and double binding assay. Application of this bispecific monoclonal antibody (BsAb) is *in vitro* effector: target cell conjugate assay by using two color cytometric analysis, we found that anti-HBx/anti-CD3 BsAb significantly enhanced the effector : target cell conjugate formation. In *in vivo* study, BsAb retargetting effector cells were more effective than that of effector cells alone in shrinkage of HCC xenografts in nude mice, not only in freshly inoculated tumors but also in established growing tumors ($p < 0.01$, $p < 0.01$). Besides, the apoptotic cells detected by *in situ* end-labelling technique were remarkable in tissues treated by BsAb plus effector cells. Pronounced infiltration of lymphocytes in the peripheral of tumor nodules can also be seen in the tissues treated by BsAb plus effector cells, but not in the controls. The results indicate that shrinkage of tumors in nude mice with therapy of BsAb retargetting effector cells was partly by initiation of apoptotic cell death in HCC cells.