Abstracts of Oral Papers

Cancer therapy with antibody-cytotoxic agent conjugates

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Extensive clinical research has already been performed to explore the concept of using monoclonal antibodies (MAbs) which recognize tumor-associated antigens to target cytotoxic agents to tumors. Although good tumor localization has been seen for many such conjugates, few significant responses have been achieved. The most important issues and technical challenges in the development of successful cancer therapies based on MAb-cytotoxic agent conjugates are:

(1) identifying suitable tumor-associated antigens;
(2) identifying and harnessing suitable cell-killing agents;
(3) achieving adequate biodistribution and pharmacokinetics;
(4) achieving adequate tumor uptake;
(5) overcoming human immune responses against rodent MAbs and against cell-killing agents attached to them.

Celltech and American Cyanamid Corp. have been collaborating for many years on the development of technology to address these issues, using both low molecular weight drugs and radio- isotopes as the cell-killing agents. Three conjugates are in advanced preclinical development. All three involve humanized rodent MAbs to minimize immunogenicity. Two of the conjugates involve the highly potent DNA-cleaving agent, calicheamicin, as the cell-killing agent, and the third involves the radioisotope ^{90}Y. All three of the antibodies internalize efficiently into cells expressing the cognate antigen.

The calicheamicin conjugates are intended for treatment of acute myelogenous leukemia (AML) and ovarian/lung cancer respectively, and the ^{90}Y conjugate for treatment of colorectal cancer. The calicheamicin conjugates give very potent antibody-targetted killing of cells expressing the cognate antigens *in vitro*, and also in mouse xenograft models at clinically-achievable doses and with good therapeutic ratios. The conjugate for AML also gives antibody-targetted killing of leukemic blast cells from the bone marrow of AML patients at low doses. The ^{90}Y conjugate gives complete eradication of tumors expressing the antigen in a mouse xenograft system with relatively low doses of radio- activity. A biodistribution study in ovarian cancer patients is presently underway with the antibody intended for delivering calicheamicin to ovarian/lung tumors.

The presentation will include a summary of the key issues in designing MAb-cytotoxic agent conjugates in general, and of preclinical data with the three conjugates in development. Results from the biodistribution study in ovarian cancer patients will also be presented.

The human monoclonal antibody SC-1 induces apoptosis of stomach cancer cells

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The human monoclonal antibody SC-1 was isolated from a patient with a signet ring cell carcinoma of the stomach by fusion of spleen lymphocytes to the heteromyeloma SPM4-0. The IgM (lambda) antibody identifies a molecule with a molecular weight of about 49 kd on stomach carcinoma cells. No reactivity was observed with carcinomas of other origin, melanomas, lymphomas or normal tissue. When tested *in vitro* the antibody inhibits tumor cell growth in both cell culture and soft agar. *In vivo* growth of stomach carcinoma cells in nu-nu mice is reduced when the antibody is injected after the tumor cells. Ultrastructural and functional studies revealed that the SC-1 antibody induces apoptosis of tumor cells.

Clinical evidence that the human monoclonal anti-idiotypic antibody 105AD7 delays tumor growth by stimulating anti-tumor T-cell responses

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A human monoclonal anti-idiotypic antibody (105AD7), which mimics a colorectal tumor-associated antigen (791Tgp72) has been developed. A Phase I trial in advanced colorectal cancer patients showed that 105AD7 was non-toxic and that immunized patients had increased survival when compared with a contemporary group of patients treated in the same centre. These encouraging results are currently being confirmed in a double blind randomized study in a similar cohort of patients. There is accumulating clinical evidence that 105AD7 delays tumor growth by stimulating anti-tumor T-cell responses. Stimulation of helper T-cells was exemplified in the phase I study as 105AD7 immunized patients showed antigen specific T-cell blastogenesis responses and enhanced IL-2 production. Further evidence was obtained from the new clinical study in which colorectal cancer patients were immunized prior to tumor resection. Immune infiltrating cells were analysed by immunohisto-chemistry and effector cell function was studied in immune cells from peripheral blood and tumor-draining lymph nodes. Both activated CD4 and natural killer (NK) cells were observed at the tumor site, which is of interest as NK cells are rarely found in colorectal tumors. Effector studies confirmed that NK activity was enhanced in 3/6 patients. Increased autologous tumor killing was also found in 3/4 patients and accumulation of CD8RO cells following 105AD7 immunization also suggested that CD8 T-cells were being stimulated.

Generation and characterization of a human monoclonal antibody against lung carcinoma

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Lung cancer is among the most lethal cancers in all industrialized countries. In the search for new biological therapy strategies, we have developed a human monoclonal antibody (MAb) against small cell,as well as non- small cell lung carcinomas, which show a high degree of specificity against these tumor antigens. Pure B-cells were isolated from tumor-draining lymph nodes using CD19 coated immunomagnetic beads, which were then immortalized by Epstein-Barr virus (EBV) trans-formation. ELISA on goat anti-human Ig polyvalent, A427 and the autologous tumor showed a high poly-clonal response in 5 out of the 150 clones assayed. Limiting dilution of the 2A3 clone resulted in the development of the MAb TB94, IgA1.k, which showed positivity against NCIH661, NCIH69, autologous tumor and A427 by ELISA, as well as by FACS analysis. Screening of the TB94 antibody for cross-reactivity indicated no reactivity against breast, ovary, melanoma, leiomyosarcoma and leukemia/lymphoma cell lines. The antibody failed to recognize normal MRC5 cells and FACScan indicated that it recognizes only surface tumor-associated antigens. Purification of the TB94 antibody was done on a Protein A Sepharose 4B column and 26.6 μg of the purified antibody when measured by FACS analysis against NCIH661, NCIH69, as well as autologous tumor cells showed 72%, 85%, and 19% shift in peak, respectively. Dot-blot of antigens isolated from NCIH661, NCIH69, and autologous tumor cells onto nitrocellulose paper showed reactivity with the purified TB94 antibody. In this, high recognition of the NCIH69 and NCIH661 was observed, while reactivity with the autologous tumor cell antigen was relatively weak. Western blot analysis on isolated antigens indicated that the antibody recognized a 32 kd molecular weight antigen on NCIH69 cell line and a cluster of antigens ranging from 28 to 106 kd in the non-small cell lung carcinoma cell line NCIH661. Immunohistochemistry was performed by the indirect bridged avidin-biotin (ABC) method. The lung cancer antibody clearly recognized NCIH661 cells, which were grown on slides, as well as paraffin-embedded tissue sections of patients with adenocarcinoma, bronchogenic, as well as squamous cell carcinomas of the lung. Collectively, these data suggest that the human MAb TB94 shows high specificity against lung tumor antigens and could potentially be used clinically for immunotherapy.

Comparison of two radiolabelled HMAb in cancer detection and dosimetry

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Two human monoclonal antibodies (HMAb) - an IgM (16.88) and an IgG_{3K} (88BV59) - recognize different epitopes on a tumor-associated antigen (CTAA 16.88), homologous to cytokeratins 8, 18, and 19. CTAA 16.88 is expressed by most epithelial-derived tumors including colon, breast, ovary, pancreas, prostate, and non-small cell lung cancer. Selective *in vivo* targetting by 16.88 and 88BV59 is related to their localization in non-necrotic areas of tumors, epitopic presentation at the cell surface of tumor cells but not of normal cells and the difference in vascularization between tumors and normal tissues. Experimental and clinical data obtained with these HMAbs, labelled with ^{131}I, ^{99m}Tc, ^{111}In, ^{186}Re or ^{90}Y suggest that, after i.v. administration, the calculated tumor:bone marrow ratios (3-5:1) did not translate into tumoricidal absorbed doses in the absence of autologous bone marrow support. However, the intracavitary, intralymphatic or intratumoral route results in much higher tumor:bone marrow ratios (40-80:1) and predicted absorbed doses in the range of 700-750 cGy/mCi of ^{90}Y-labelled IgM. The longer intratumoral residence time of the 16.88 compared to the 88BV59 after intratumoral administration in nude mice carrying the LS174t colon tumor xenograft suggests that molecular size may be an important consideration in selecting a MAb for radioimmunotherapy. Both radiolabelled antibodies have been administered intravenously or subcutaneously to over 250 patients without evidence of a human antihuman antibody (HAHA) response. They appear well suited for repeat administration as diagnostic or therapeutic agents.

Initial characterization of a human MAb, derived from unimmunized normal peripheral blood lymphcytes, reactive against adenocarcinoma cells

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Peripheral blood lymphocytes from a healthy donor were fused, without *in vitro* immunization, with GM4672B cells and grown in HAT medium. Hybrids secreting >500 ng/ml of IgG or IgM were expanded and cloned. Clone P4D2B was selected on the basis of high immunoglobulin production and high proliferation rate. It produces IgM-$\underline{k}$ at a rate of 790 ng/ml per 10^6 cells/24 h. The antibody was purified and reactivity with a variety of cell lines was determined by immunocytochemistry. Intense cytoplasmic staining was found in Calu-3 lung adenocarcinoma cells, LS-174T and HCT-8 colon adenocarcinoma cells but not in breast cancer cells (MCF-7), squamous cell carcinoma cells (Calu-1) and a variety of lymphoid tumor cell lines. The molecular size of the antigen is between 66 to 97 kd, and is present in the cytosol and detergent soluble membrane fractions. The antigen is formalin-labile but stable in paraformaldehyde or acetone fixation protocols and is not related to low molecular weight keratin or vimentin. The ability of normal lymphocytes to generate tumor-reactive monoclonal antibodies is a phenomenon that requires further research and suggests caution in the use of *in vitro* immunization protocols for the development of human monoclonal antibodies (MAbs). Unstimulated, unimmunized control fusions must be carried out to prove that tumor-specific *in vitro* immunization is successful.

Neutralizing antibodies against HIV-1 from combinatorial phage display libraries

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Combinatorial phage display libraries were prepared from long-term HIV-1 seropositive survivors (>12 years). In initial experiments, monomeric HIV-1 envelope subunits were used for enrichment and screening of clones (*1*). Selection for potency and strain cross-reactivity was achieved through experimental design. Library donors were US males, presumably infected with a clade B strain of HIV-1, whereas the antigen for affinity selection was recombinant gp120 IIIB, thereby favoring selection of cross-reactive antibodies. One clone, b12. was found to be exceptionally potent in its capability to neutralize HIV-1; laboratory strains HIV-1 MN and IIIB were neutralized in the nanomolar range. The majority of antibodies obtained, however, were far less potent even though they were also directed against the CD4 binding site, competed with b12 for binding to recombinant gp120, and had affinities similar to b12 (*2,3*). Recent experiments have shown that conversion of b12 into a whole IgG1 molecule (IgG1 b12), yielded an antibody capable of neutralizing a large proportion of primary isolates of HIV-1. IgG1 b12 neutralized more than 75% of primary isolates tested at physiologically relevant concentrations (*4*). The observation that a single antibody is capable of this feat has important implications for vaccine research. Evidence from antibody binding to infected cells suggests that b12 may recognize a native conformation of gp120 more effectively than other antibodies directed against the CD4 binding site (*5*). With this observation in mind, we set out to perform panning of the antibody phage display libraries against HIV-1 envelope glycoproteins in native (multimeric) configuration. Tetrameric molecules purified by sucrose gradient centrifugation from a recombinant gp120-gp41 produced in a vaccinia expression system were analysed most extensively. Most antibodies obtained were from the non- neutralizing type as found after panning of these libraries against the monomeric gp120 and gp41 subunits. Several new antibodies were obtained, however, and their characteristics and neutralizing ability will be discussed.

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Cloning and biological characterization of human single chain Fv fragments that mediate neutralization of HIV-1 *in vitro*

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HIV-1 is the etiological agent of the acquired immune deficiency syndrome (AIDS) and related disorders. Among the different strategies adapted to prevent AIDS, passive immunization has shown promise as an effective method to slow the progression of HIV infection in adults, as well as in children. Monoclonal antibodies are the immunological reagents of choice for passive protection because of their monospecificity and general lack of toxicity. In the present study, we have developed a simple phagemid vector for cloning of human antibody-like binding proteins. Using this vector, we have generated a large panel of human antibody Fv fragments from an asymptomatic seropositive HIV-1 by affinity selection from an antibody library expressed on the surface of filamentous phage. Human Fv fragments as a single chain containing variable regions of heavy and light chains were successfully expressed in this vector. These cloned Fv fragments were evaluated for binding to the HIV-1 external membrane glycoprotein (gp120). Those recombinant clones which bound to HIV-1 gp120 were examined for their ability to neutralize infection by different isolates of HIV-1. Neutralization was determined by the ability of the Fv fragment to inhibit syncytia formation using MT-2 cells. Several of the clones were able to effectively neutralize infection by HIV-1 IIIB. In addition, we have obtained broad mapping data using vaccinia constructs which express truncations of the HIV-1 envelope glycoprotein. This strategy of cloning resulted in the development of functional antibody reagents with different anti HIV-1 biological properties *in vitro.* These recombinant Fv fragments have potential utility as immune reagents as well as in the design of potential immuno-therapeutics. Further biological and molecular characterization of these clones is in progress.

Potency of various human monoclonal antibodies in neutralization of HIV-1 primary isolates and lab strains

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The envelope protein of HIV-1 is known to possess at least five regions which induce neutralizing antibodies in humans during infection. Monoclonal antibodies (MAbs) against three neutralizing epitopes V2, V3 and the CD4-binding domain (CD4bd), were generated from lymphocytes derived from HIV-1 infected subjects by EBV transformation followed by fusion with a heteromyeloma. All MAbs were purified by Protein A chromatography and tested in neutralization assays against 10 primary isolates and 3 laboratory strains. In an assay using PHA-stimulated PBMC, all primary isolates from clade B were neutralized by human MAbs in the range 1.0-12.5 μg/ml and 2.0-75.0 μg/ml for 50% and 90% neutralization, respectively. The neutralizing potencies of the three categories of human MAbs against primary isolates were very similar, although mechanisms of neutralization for each type may differ. In contrast to the primary isolates, the neutralizing abilities of the same MAbs against laboratory isolates, propagated in T-cell lines, were different. Anti-V3 MAbs were 50-fold more effective than anti-CD4bd MAbs and the anti-V2MAbs displayed no neutralizing activity. Thus, anti-CD4bd MAbs are equally effective in neutralizing primary and laboratory strains, the anti-V3 MAb tested is much more potent against laboratory strains and the anti-V2 MAb neutralizes only primary isolates. The differences in the potency of neutralizing human MAbs is related to the virus used and the assay format, in which the type of target cell (T-cell line vs. PBMC) and the cells' activation state play an important role.

Human monoclonal antibodies against a conformational domain of cytomegalovirus glycoprotein B

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Human cytomegalovirus (CMV) causes significant morbidity and morbility in immunocompromised and AIDS patients. Twenty percent of the AIDS patients suffer from CMV-retinitis which leads to blindness if it is not treated. *In vivo* diagnostics using fluorescein-conjugated human anti-CMV antibodies could be employed to detect CMV-retinitis of AIDS patients. We generated human anti-CMV antibodies from spleen cells of a CMV-seropositive patient. The selected antibodies (subclass: IgG3- lambda- light-chain) bound specifically to glycoprotein B of CMV as shown by immune precipitation and indirect immuno-fluorescence. Immunofluorescence was performed with human astrocytoma transfectants expressing glycoprotein B (provided by Prof. Radsak, Marburg). The antibodies did not recognize a linear sequence of glycoprotein B in immunoblots. To analyse further the antigenic region recognized by the antibodies, the extracellular and trans-membrane domains of the glycoprotein B gene were expressed as a ß-galactosidase fusion protein in BHK cells. Specific binding of the human antiglycoprotein B antibodies to the extracellular domain of glycoprotein B was demonstrated by immunofluorescence. Additionally we are purifying glycoprotein B (antigen for a subunit vaccine) by affinity chromatography using the human anti-glycoprotein B antibody. Furthermore, the genes encoding the human antiglycoprotein B antibody were cloned and expressed as Fab fragment in *E. coli.*

Neutralizing human monoclonal antibodies to RSV fusion protein isolated from hu-SCID mice

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Two human IgG_1 , $\underline{k}$, monoclonal antibodies to respiratory syncytial virus (RSV) fusion (F) protein were isolated from hu-SCID mice. Selected hu-SCID mice that had high titers of antibodies (10^6 to 10^7) to the immunizing antigen, were sacrificed and human lymphocytes were washed out from the peritoneal cavity. These cells were cultured in 96 well plates. Supernatants were tested for presence of antibodies to the immunizing antigen and these cells were immortalized. Two antibodies, Virab-1 and Virab-2, specifically recognized RSV-infected HEp-2 cells in FACS, but not uninfected HEp-2 cells or cell lines representing liver, prostate and lymphocytes. Both antibodies have *in vitro* neutralizing activity to a broad range of virus isolates, covering both strains A and B as well as wild type and laboratory strains. The concentration of antibody resulting in 50% infection neutralization of 100 pfu of RSV in the presence of complement was < 30 ng/ml. The dissociation constant for both antibodies was $< 10^{-9}$ M.

The pathogenic human monoclonal anti-DNA 1/16 that induce experimental systemic lupus erythematosis in mice is encoded by the VH4.21 gene segment

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Systemic lupus erythematosis (SLE) can be induced in mice by immunization with a human anti-DNA IgM monoclonal antibody (MAb), that expresses the common idiotype (Id) designated 16/6 Id. We have been successful in inducing experimental SLE using the 16/6 Id MAb and murine anti-16/6 Id MAb, as well as monoclonal antibodies with parallel Id, e.g. anti-La and anti-cardiolipin. The original human hybridoma 16/6 formerly secreted an IgM antibody that bound ssDNA and carried the 16/6 Id, had switched in culture to secrete an IgG molecule. Herein we show that the IgG 16/6 MAb expresses the 16/6 Id and is capable of inducing experimental SLE in susceptible mouse strains. The identity of the IgG 16/6 anti-DNA MAb to the original IgM one was shown both by serological techniques and on the T-cell level. Thus, lymph node cells of mice immunized with either the 16/6 human IgG MAb, or a murine 16/6 Id bearing MAb, proliferated in the presence of both antibodies. T-cell lines specific for the IgM 16/6 MAb react specifically to the IgG 16/6 MAb. The human IgG 16/6 MAb was found to be encoded by a germ line gene from the human VH4 gene family, with high similarity to the germ line gene VH4.21 that was shown before to code for anti-DNA antibodies isolated from SLE patients. The VH4.21 germ line gene was found to code also for most antibodies with cold agglutinins activity that were isolated from patients with cold agglutinin disease. Interestingly, the patient from whom the original 16/6 Id was derived also had a cold agglutinin disease. It can be concluded that the isotype switched human anti-DNA MAb, carrying the 16/.6 Id is identical in its pathogenic potential to the original hybridoma secreting IgM anti-DNA antibody. The IgG 16/6 Id MAb is encoded by a germ line gene of the VH4 family, members of which were found to code for other DNA-specific antibodies of SLE patients.

Mapping of therapy-induced antibodies to interferon-alpha 2

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Human interferon alpha-2 (IFNα2) has been approved for therapeutic application in a range of human malignancies and viral diseases. However, this therapy can be complicated by the development of antibodies to IFN. Until now the immunogenic structures of human IFNα2 responsible for eliciting of antibodies in man were not identified. Therefore, we focused our study on elucidation of this important question. Ten patients with hepatitis B were treated with recombinant human IFNα2 and the specific anti-IFNα2 activity was detected in sera for two of them. To localize the epitopes recognized on IFNα2-molecule by these human antibodies the competitive RIA was employed. Positive sera competed with a panel of mapped murine monoclonal antibodies to recombinant IFNα2 for binding of radiolabelled recombinant human IFNα2. Monoclonal antibodies were specific for six different epitopes which were located on both N-terminal and C-terminal parts of IFN-molecule. Only monoclonal antibodies with epitopes in the N-terminal half inhibited binding of the therapy-induced antibodies with IFNα2. Our result indicates that for the immunogenicity of human IFNα2 in man is crucial for the domain formed by residues 30-53.

Dual recognition of lipid A and DNA by human antibodies encoded by the V_H4-21: a possible link between infection and lupus

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The V_H4-21 gene segment encodes the vast majority of anti-red cell autoantibodies of I/i specificity, and recognition of red cells appears to occur via a sequence in the framework region (FWR). The gene is also used by a subset of anti-ssDNA and anti-dsDNA antibodies in patients with SLE. In this case, recognition of DNA appears to be more conventional, being mediated via the CDR3 region and involving sequences of basic amino acids, particularly arginine. Although the expressed V_H4-21 gene therefore has potential for interaction with two autoantigens, the basic sequences in CDR3 inhibit the ability of the FWR to interact with the red cell antigen. The V_H4-21 gene segment also encodes IgM antibodies against bacterial lipid A, and we have investigated the pattern of reactivity of sequenced IgM antibodies with the exogenous antigen as compared to red cell antigen or DNA. Antibodies monospecific for red cell antigen (10/10) failed to react with lipid A; however, antibodies which recognized DNA (4/5) bound to lipid A with high (2/4) or moderate (2/4) avidity. All the anti-lipid A V_H4-21 sequences were unmutated, with basic amino acids in CDR3 demonstrating common avidity-associated motifs. Results indicate that, for the V_H4-21 gene, there is dual recognition of a bacterial antigen and DNA, which may link infection to SLE.

Human monoclonal anti-Rh antibodies produced by human-mouse heterohybridomas express the Gal α1-3 Gal epitope

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The clinical use of murine monoclonal antibodies in man has been hampered by a well documented immune response elicited by these proteins when administered *in vivo*, the so-called human anti-mouse (HAMA) response. More recently, the recognition of the Gal α1-3 Gal epitope on mouse monoclonal antibodies by 'natural' human anti- Gal antibodies has been postulated as responsible for an accelerated removal of mouse monoclonal antibodies intended for therapeutic use when administered in the human body, even before the appearance of the HAMA response (Borrebaeck CAK *et al. Immunol Today* 1993; **14:** 477). To solve the previously mentioned problems, techniques for the production of human monoclonal antibodies have been developed in many laboratories all over the world. In this work, the presence of the Gal α1-3 Gal structure (Gal epitope) in the carbohydrate component of 15 human monoclonal antibodies with specificity for the Rh blood group factor and produced by human-mouse heterohybridomas was evaluated. To do that, an anti-globulin-like agglutination test and an enzyme linked immunosorbent assay were performed using an affinity-purified anti-Gal antibody obtained from the serum of an AB blood group donor. Using the antiglobulin reaction, results were obtained showing that only five of the fifteen human monoclonal antibodies tested contained the structure at levels sufficient to allow agglutination. However, all 15 monoclonals were positive using the more sensitive enzyme-linked immunosorbent assay. By means of an indirect immunofluorescence assay, the same anti-Gal antibody was used to test the presence of Gal epitopes on the surface of the producer heterohybridomas. Twelve of the fifteen hybridomas studied expressed the Gal structure on its surface. It is concluded that human monoclonal antibodies produced by human-mouse heterohybridomas may express in a variable degree the Gal epitope and this should be a consideration when selecting these monoclonals as reagents for therapeutic applications.

The Fc receptor III-mediated functional activity of human red cell antibodies is affected by the extent of galactosylation and by sequence changes in the CH2 domain of IgG

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Human monoclonal antibodies (MAbs) to the Rh D blood group antigen were produced by EBV-transformed B cell lines grown in serum-free medium at either high (HD) or low (LD) cell densities. The percentage of agalactosyl IgG(%G(0)) was determined by the binding of a MAb (GN7) to terminal GlcNAc. The LD MAbs had very low levels of agalactosyl IgG (less than 5%) whereas the %G(0) was higher in the HD MAbs (about 10%). Aliquots of BRAD-3 (IgG3) and BRAD-5 (IgG1) monoclonal anti-D (LD) were digested with ß-galactosidase from *Streptococcus* strain 6646K. The %G(0) in these MAbs after galactosidase treatment was 20% and 30% respectively. Red cell autoantibodies were eluted from red cells of 24 patients with autoimmune hemolytic anemia, and were found to have a very wide range of %G(0) levels. Functional interactions of IgG with FcγRIII were determined by measuring the lysis of red cells by K cells in antibody dependent cell-mediated cytotoxicity (ADCC) assays. In three experimental situations, (i) MAbs produced at high or low cell density, (ii) MAbs before and after digestion with galactosidase , and (iii) the purified autoantibodies, it was found that IgG with high levels of galactose (i.e. low agalactosyl IgG) promoted more hemolysis in ADCC assays than IgG with less terminal galactose. It was observed that while two IgG1 anti-D MAbs (BRAD-5 and JAC10) had similar galactosylation, their effector function was different; JAC10 exhibited markedly reduced activity in the K cell ADCC compared to BRAD-5. cDNA spanning the hinge and CH3 regions was isolated from the cell lines, amplified using PCR and sequenced. It was found that JAC10 had residue changes at positions 233 and 239, which flank one of the putative FcγRIII interaction sites, whereas the sequence of BRAD-5 matched that published for IgG1. Since red cells coated with non-complement binding IgG antibodies are destroyed *in vivo* by FcγR-bearing macrophages in the spleen, these results are relevant to the choice of monoclonal anti-D for prophylaxis against Rh D hemolytic disease of the newborn, and for understanding the pathogenicity of auto-antibodies.

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Characterization of human antibody variable fragments against U1RNA-associated autoantigens, isolated from a synthetic- and a patient-derived combinatorial library

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We have characterized human antibody fragments against U1RNA-associated autoantigens from a synthetic combinatorial library and a patient-derived combinatorial spleen library. Using phage technology, autoantigen binding Fab fragments were isolated from a large human synthetic combinatorial Fab library (titer 6.5 x 10^{10}), containing all human heavy and light chain germline genes with synthetic ran- dom CDR3 domains (*1*). From this library, 3 different Fab clones (A1-A3) were selected against the U1RNA-assoc- iated A protein (U1A) and 1 Fab clone was isolated against U1RNA-associated C protein (U1C). Both these U1RNA- binding proteins are involved in pre-mRNA splicing and autoantibodies against these proteins are often present in patients with SLE and SLE-overlap syndromes (2). The first combinatorial library derived from a spleen of an auto-immune patient, was also screened with the U1A- and U1C autoantigen. A single-chain variable fragment (scFv) against U1A(sp1) was isolated from this IgG library. VH and VL genes were sequenced and germline gene usage was determined. The isolated Fabs and scFv react specifically with recom- binant proteins in ELISA and on Western blot with U1A out of a HeLa total nuclear extract. With protein-A-coupled Fabs A1 and A3 and scFv sp1, we were able to precipitate U1 ribonucleoprotein complex (U1RNP) out of ^{35}S-methionin labelled HeLa nuclear extract. Epitope mapp-ing was performed with *in vitro* translated wt and N- or C-terminal truncated proteins. In the case of U1A two differ-ent epitopes are recognized by different clones. Clone A2 recognizes the C-terminal end of U1A (aa 203-282) and cross-reacts with B", a U2RNA-associated autoantigen which is 95% identical in this C-terminal domain with U1A. The other two anti-U1A Fabs, A1 and A3, as well as the scFv sp1, recognize the central domain of U1A(aa 118-203), and do not cross-react with B". The heavy chain of anti-U1A sp1 clone uses the germline gene DP65 and has 8 somatic mutations randomly distributed throughout the variable region. Two of the three anti-U1A Fabs(A1 & A3) use the same germline gene for their heavy chain, and recognize the same epitope on U1A. In normal individuals, the DP65 gene (VH4 family) is only used in ~1% of the heavy chain sequences. This suggests that for U1A this synthetic library mimics the *in vivo* autoantibody repertoire.

Isolation of human recombinant anti-Rhesus D antibodies from a hyperimmunized donor

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The prophylactic efficiency of anti-Rhesus D IgG for prevention of hemolytic disease of the newborn is well established. However, current methods of production are associated with various risk factors and technical problems such as the use of blood products with attendant safety problems and repeated immunization of donors for production of higher teter antiserum. The construction of recombinant antibody libraries using bacterial expression vectors and subsequent Fab display on filamentous phage particles offers an alternative means of producing such antibodies. We have used this technique to construct a recombinant library from a hyperimmunized donor who was boosted i.v. with Rhesus D+cells. At +5 days peripheral mononuclear cells were harvested, RNA was extracted and used for PCR and library construction. Phage expressing Fab (Phabs) were selected on whole red blood cells in a two-step procedure: first, absorption with Rhesus D negative cells followed by selection and elution with Rhesus D positive cells. The resulting eluted Phabs were screened for agglutination of Rhesus positive red blood cells in an indirect hemagglutination assay using a rabbit anti-phage antibody as crosslinking antibody. Positive agglutination was seen after the third round of selection. After a further cloning, step-soluble Fab was produced in *E. coli.* This total Fab population, purified on a hydroxylapatite column, when tested in an indirect hemagglutination assay shows specificity for the Rhesus D antigen with no reactivity against other common blood group antigens. Individual clones from the library have been sequenced indicating an oligoclonal population with VH genes confined to the VH3 family. These recombinant Fab antibodies will provide the basis for a new generation of therapeutic reagents.

Human MAbs reactive with public determinants on HLA class 1 antigens

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Many serological studies on allospecific sera have shown the existence of public determinants to allelic HLA A-locus or B-locus products. A precise definition of such public determinants is difficult because virtually all allosera contain mixtures of antibodies. We have established a number of human hybridomas producing specific antibodies reactive with public determinants. Peripheral blood lymphocytes from parous women whose sera contained a variety of HLA alloantibodies, were EBV transformed and cultured in the presence of an anti-CD40 MAb immobilized on CDw32 transfected L-cells as well as interleukins. We electrofused antibody producing EBV cell lines with a heteromyeloma cell line and analysed the resulting HuMAbs for HLA reactivity against a large panel of HLA typed cells. Previous studies have shown that this method permits the generation of HuMAbs against private determinants. The present study deals with the specificity spectrum of HuMAbs against public HLA class I determinants. Of particular interest was a collection of 23 Abs generated from the lymphocytes of a primiparous woman whose child carried the A3,B70 paternally derived haplotype. Most of the HuMAbs were reactive with determinants on the HLA-A3 molecules including those reacting with [A1,A3,A11,A36,A32], [A3,A11,A32], [A1, A3, A11,A24],[A1,A3,A36,A26,A11,A29,A30,A31,A33], HuMAbs, reactive with B-locus public determinants were also produced. Some of these allele combinations share common amino acid residues suggesting that such residues are critical in forming these public epitopes. For example, the epitope responsible for binding of HuMAb OK2H12, reactive with a determinant shared by [HLA-A1,A3,A11, A32] is shaped by residues 62Q and 66N on the α2 domain, but simultaneous presence of 56R (as in A30 and A31) prevents the recognition by MAb OK2H12. Similarly, the epitope seen by HuMAb OK5A3, on [HLA-A1,A3,A11, A24] is shaped by 142I, 144K,145R on the α2 domain, but binding is abrogated when residue in position 144 is substituted by a Q. These and other examples demonstrate that HuMAbs of this kind can be used for epitope mapping with the presently available amino acid sequences. We propose that these HuMAbs can be used as public epitope typing reagents for matching organs and transplant recipients.

Isolation and characterization of autoantibodies from patients with autoimmune thyroid disease using phage display combinatorial libraries

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Hashimoto's thyroiditis and Graves' disease are at either end of a spectrum of autoimmune conditions affecting the thyroid gland. Hashimoto's thyroiditis is characterized by a deficiency in thyroid activity and a high serum titer of autoantibodies to thyroglobulin (Tg) and thyroid peroxidase (TPO). Graves' disease is associated with overactivity of the thyroid gland. Anti-thyroglobulin and thyroid peroxidase antibodies are again present but of clinical importance are antibodies to the thyroid stimulating hormone receptor that artificially stimulate the gland to produce hormones. In this study we aim to look at and compare the antibody repertoire in representative patients with Hashimoto's thyroiditis and Graves' disease using phage display combinatorial libraries. Thyroid lymphocyte RNA was used to create a library in the phagemid vector pComb3. Previously we have reported the isolation of three anti-TPO Fabs and one anti-Tg Fab from this library. All four were shown to bind with high affinity (~ 10^9 M) and specificity for their antigen (Hexham JM, *et al. Autoimmunity* 1994;**17:** 167-179). Recently we have isolated at least one other Fab binding specifically and with high affinity (5 x 10^8 M^{-1}) to Tg. This Fab, which we have named 1K, has also demonstrated inhibition of serum autoantibodies indicating that it is recognizing an epitope associated with the disease. Sequence analysis has allowed assignment of germline genes. The light chain originates from the germline kv325 which was also found in our other anti-Tg Fab and in one of our anti-TPO Fabs. The heavy chain is derived from the VH26 germline gene with $D_{LR}1$ encoding the D segment and J_H6 encoding the J segment. Both chains have undergone significant somatic mutation indicative of an antigen driven response. Other Fabs which bind to Tg have also been isolated and these are currently under further investigation.

This work is now being extended to look at a patient with Graves' disease. Thyroid lymphocyte RNA has been isolated and used to construct a phage display combinatorial library of IgG1 heavy and *k* light chains in pComb3HSS. This library is being screened for Fabs binding specifically and with high affinity to thyroid autoantigens.

Phage antibodies as useful immunochemical reagents

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The display of repertoires of antibody fragments on the surface of filamentous bacteriophage offers a new way of making antibodies with predefined binding specificities. We explored the use of this technology to make immunochemical reagents to a range of antigens by selection from a repertoire made *in vitro* from human V-gene segments. From the same 'single pot' repertoire, phage were isolated with binding activities to antigens, including the intracellular proteins p53, elongation factor EF-1*a*, immunoglobulin binding protein, rhombotin-2 oncogene protein and sex determining region Y protein. Both phage and scFv fragments secreted from infected bacteria were used as monoclonal and polyclonal reagents in Western blots. Further- more, the monoclonal reagents were used for epitope mapping (a new epitope of p53 was identified) and for staining of cells. The affinities of the antibodies isolated from primary phage repertoires of 10^8 clones are only moderate (about 10^{-6}M). A further diverse repertoires of human heavy and light chains were recombined in bacteria, generating a larger Fab repertoire of 6.5 x 10^{10} clones with binding affinities for haptens in a nM range. This shows that antibody segments with both moderate or high affinities for research can be readily derived from 'single pot' phage display libraries.

A new phage-display system to construct multi- combinatorial libraries of very large antibody repertoires

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We present a novel method of constructing large antibody repertoires through the recombination of two separate heavy (V_H) and light (V_L) chain gene libraries. The process, which makes use of lambda phage *att* recombination sites, leads to the irreversible physical association between V_L and V_H sequences carried respectively by a plasmid and a phagemid. The heat-shock induced expression of the Int recombinase allows perfect control of recombination. Selection of the recombinant phagemid is made possible by the assembly, *in vivo*, of a new genetic marker (chloramphenicol or gentamicin resistance) created only after the correct recombination event. The functionality of the system has been demonstrated by the display of a chimpanzee anti-HIV gp160 Fab. Theoretically, all possible recombinations between light and heavy chain sequences should be obtained and it should be possible to generate multicombinatorial libraries of close to 10^{12} clones. Initial results in the construction of such libraries will be presented.

Chain shuffling: a powerful tool for the manipulation of human antibodies

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Antibodies with predefined specificities can be selected from repertoires displayed on filamentous phage by fusion to a minor coat protein. Mediate size libraries can be created by direct cloning of randomly combined V-gene pairs; very large repertoires can be constructed by combinatorial infection. Besides these novel technologies, we have developed complimentary methods based on chain shuffling to improve the affinity or specificity of antibodies selected from primary repertoires, or to convert rodent antibodies into completely human antibodies with similar binding characteristics. In chain shuffling, one of the two antibody variable domains is replaced with a repertoire of naturally occurring variants (derived from PBL mRNA) and the shuffled combinations selected on antigen. The affinity of an anti-hapten antibody, originally isolated from a primary human antibody repertoire was matured 300-fold in a model experiment (Marks *et al. Bio/Technol* 1992; **10:** 779). The same approach was applied to affinity mature an anti-V3 loop antibody of high affinity (10^{-10}M). The affinity improvement was in the region of 30-fold when measured on MN gp120; the affinity for variant loops was also enhanced (unpublished). Last, a human anti-human protein antibody was affinity matured 10,000 fold.Thus, in the absence of specific structural information, the affinity of antibodies can be improved to very high levels by shuffling with natural V-gene pools; the degree of improvement depends on the affinity of the starting antibody. Chain shuffling was also applied to convert animal into human antibodies. In a model experiment, the light chain from a murine anti-hapten antibody with known structure was shuffled sequentially with repertoires of human partner chain domains (Figini *et al. J Mol Biol* 1994; **239:** 68). The selected heavy chain partners retain many critical antigen binding features found in the original murine heavy chain. The same approach was used to convert a murine anti-TNF antibody into a human version (Jeepers *et al. Bio/Technol* 1994; **12:** 899); the full human antibody has a similar affinity and the same *in vivo* activity (enhancement of anti-tumor effect of TNF) as the murine antibody. Thus, chain shuffling of antibody V-genes with natural diverse V-gene pools provides a powerful tool for the creation of affinity improved antibodies and for the 'species conversion' of antibodies. Together with the diversity in binders offered by selection from large primary antibody phage repertoires, the isolation of human antibodies binding with a particular kinetic behavior and with specificity for any chosen antigen should now be feasible.

Phage-displayed antibodies specific for a cytoskeletal antigen: selection by competitive elution with a monoclonal antibody

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A phage display library of V_L and V_H sequences of mouse antibodies was constructed, which contained 4.5×10^7 independent clones. From this library pool of phages were selected up to four biopanning rounds on cytoskeletal preparations of ovarian carcinoma cells (OVCAR-3). Pahge of these pools were then allowed to bind to a cytoskeleton preparation of bladder carcinoma cells (T24). Binding phage were challenged by a monoclonal antibody (MAb) directed against an epitope on cytokeratin 8. Displaced phages were rescued and screened for anti-cytokeratin immunoreactivity by ELISA, indirect immunofluoresence and Western blotting. About 50% of selected library can be used to obtain human scFv-s against epitopes already defined as valuable diagnostic markers by mouse MAbs.

A novel positional humanization method generates human consensus frameworks: humanized BrE-3 and Mc3 for breast cancer therapy

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We used a positional consensus method in the previous humanization of the two murine antibodies BrE-3 and KC4G3. The method dictates that residues in certain conserved positions must be retained in order to guarantee the preservation of the original binding properties. Indeed these previous humanizations were completely successful. These frameworks were 90-97% identical to 'ideal' human consensus frameworks. Nevertheless, we speculated that the positional consensus may be unnecessarily too conservative with respect to the preservation of residues with inwardly pointing sidechains. We speculated, in addition, that the conservation of this class of residue may still cause the humanized antibody surface to have an immunogenic shape due to the distortion caused by the residue buried directly underneath. Thus, we eliminated 18 and 13 'buried' residues from the positional consensus of the VL and VH frameworks, respectively. The shorter positional consensus sets comprise solely those residues that have a high probability of contacting either the CDRs or the opposite chains. We applied the novel positional consensus to the humanization of antibody MC3 with complete success. We mutated the original murine V_kV and V_H IIA frameworks to reflect the consensus sequences for human V_kIV and V_H I subclasses. Three positions in V_k and 13 positions in V_H were left intact. All others were changed from murine to human. The similarity with the respective human consensus frameworks increased from 77 to 96% for VL and from 69 to 85% for VH . Many human antibodies show more differences from their own consensus sequences than HuMC3V_H does. Eg, we found human V_H I frameworks with as many as 29 differences from their own consensus sequence. Two of the three antibodies that we humanized, BrE-3 and MC3, are highly relevant for breast cancer therapy. Murine BrE-3, in particular, has been tested in human clinical trials with great success although it elicited a HAMA response. MC3 has been tested in animals carrying transplantable MX-1 tumors and demonstrated an extraordinary specific tumor uptake. Results from biodistribution and therapy experiments in animal models showed that the humanized versions of BrE-3 and MC3 have tumor-specific binding properties that are similar to those of their original murine counterparts. In summary, our methods have consistently generated humanized antibodies whose frameworks closely approximate those most commonly found in human antibodies, without detectable loss of affinity. [Partly supported by NIH-NCI grants RO1-CA39932 and PO1- CA42767.]

The development and characteristics of an explant procedure for investigating immune responses *in vitro*

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We have recently developed a method for the rapid preparation of uniform tissue explants from human lymphoid tissue together with procedures which permit their storage in liquid nitrogen without substantial loss of viability or functional impairment. Following *in vitro* culture cells within explants survive as well as their counterparts in single cell suspensions. There are, however, marked differences in their performance. Spontaneous immunoglobulin production in explants commences earlier and greater levels of immunoglobulin are produced. Furthermore, there are marked differences in their cytokine secreting profiles. Studies with polyclonal stimuli such as PHA indicate that cells in explants can be activated; however, approximately 5-fold higher concentrations of mitogen are required than those effective in suspension cultures. In this presentation, the basic explant technology will be described and its characteristics compared and contrasted with that of suspension cultures. We believe the explant system provides a potent tool for investigating the complex interactions involved in the generation of antibody responses *in vitro* and could lead to improved procedures for *in vitro* immunization.

Production of human antibodies by mice engineered with human immunoglobulin YACs

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Mice producing large repertoire of human antibodies in the absence of mouse antibodies were generated by introducing large, germline configuration segments of the human heavy and kappa light chain loci, contained on yeast artificial chromosomes (YACs), into the germline of mice engineered by gene targetting to be deficient in functional mouse immunoglobulin (Ig) genes. The human Ig YACs restored normal B-cell development and production of fully human antibodies in the absence of mouse antibodies. The generated mice were shown to produce a diverse, adult-like repertoire of fully human antibodies at levels approaching those observed in normal serum. Upon immunization with different antigens, including human antigens, these mice mounted an antigen-specific human antibody response. These mice have been utilized to generate antigen-specific fully human monoclonal antibodies. The generated mice are being exploited to develop fully human therapeutic monoclonal antibodies.

Use of miniaturized hollow fiber bioreactor for the development of human monoclonal antibodies from a patient with B-chronic lymphatic leukemia

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Peripheral blood lymphocytes (PBLs) of a 64-year old patient with a B-chronic lymphatic leukemia (B-CLL) in stage IV of the disease were cultured successfully in a miniaturized hollow fiber bioreactor over a period of 8 weeks in protein-free medium. In the harvests interleukin-3 and high concentrations of immunoglobulin could be found. The established B-CLL cell population was fused afterwards with the human-mouse-heterohybridoma cell line CB-F7 in order to develop human monoclonal antibody (HMAb) producing cell lines. All growing and producing clones of this fusion were analysed by PCR techniques. At least one monoclonal IgM-producing cell line could be established and analysed in comparison with the antibodies found in the harvest of the bioreactor. The operation of miniaturized high cell density bioreactors for the development of HMAbs for the possible treatment of B-CLLs will be discussed.

Keywords: miniaturized hollow fiber bioreactor; human-mouse-heterohybridoma; B-CLL; long-term cultivation

New approaches to human monoclonal antibody production: use of transgenic mice

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As the limitations of murine MAbs for therapy have become apparent, a number of different approaches have been taken to minimize their immunogenicity, enhance their effector function and extend their half-life in man. While many of these approaches have reduced the immunogenicity of murine sequence MAbs, the ideal reagent would be an antigen-specific, high affinity human MAb. To this end, we have chosen to inactivate the endogenous murine heavy and kappa (*k*) light chain immunoglobulin genes and to incorporate into the mouse genome mini-loci encoding unrearranged human heavy and *k* light chain immunoglobulin genes. The resultant HuMAb transgenic mice have B-cells which express a human Ig receptor, develop in the bone marrow and populate peripheral lymphoid organs. The sera of these HuMAb mice contain human IgM, IgG and Ig*k* in the absence of murine IgM and Ig*k*. In response to challenge with a variety of different immunogens, an initial IgM response is observed followed by seroconversion to an IgG response concomitant with somatic mutation. HuMAbs directed against human antigens have been derived from immunized transgenic mice by standard hybridoma techniques. Aside from the nature of the MAb itself, hybridomas produced from the HuMAb transgenic mice show properties that are not significantly different from the wild type mice. The HuMAbs produced from these transgenic hybridomas have been shown to be antigen-specific, biologically active and of high affinity. The availability of human sequence MAbs specific for human antigens allows the consideration of long term passive immunotherapy for chronic disorders such as autoimmunity, transplant rejection and cancer.

Production of human MAbs in high concentration in the modular minifermenter miniPERM™

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During recent years HuMAbs have become an important tool not only in biomedical research but also in clinical diagnosis. The preparation of human hybridomas, although still not as easy as the preparation of mouse hybridomas, is now routine in a large number of laboratories. However, the low concentrations at which HuMAbs are released by the human hybridomas make their production costly. It was for these reasons that we have tried the new modular bioreactor miniPERM™ which was recently introduced by Heraeus Sepatech for the production of HuMAbs. The miniPERM™ is a small bioreactor composed of two modules separated from each other by a dialysis membrane. Parts of the outer surface of the small (35 ml) production module as well as of the larger (600 ml) supply module are made from a thin gas-permeable silicone rubber membrane. This constructional feature allows exchange of nutrients and metabolites through the dialysis membrane and exchange of oxygen and carbon dioxide through the silicone rubber membranes. Several human hybridomas, producing MAbs (of the IgG as well as of the IgM class) specific for human red cell antigens, were cultured in the new bioreactor. So far, cells of the four lines tested could be cultured without problems. Cell densities between 14 and 30 x 10^6 cells per ml and MAb concentrations of 200 - 1000 µg/ml were obtained in our culture experiments. Hybridomas could be cultured in serum-reduced or even in serum-free media. As compared to the MAbs produced in conventional techniques, the MAbs obtained in the miniPERM™ minifermenter expressed the same properties. Their specificity as well as affinity were identical to those of antibodies originating from routine roller or spinner culture. Owing to the high immunoglobulin concentration and the culture under serum-reduced or serum-free conditions, antibodies produced in the miniPERM™ culture system provided useful materials for further purification.

Getting what you want from phage display

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Since the first demonstrations of antibody display on filamentous phage in 1990 there have been many examples of the successful isolation of human antibody genes using the system. In the ensuing four years, the usage has become more sophisticated with cell surface selection, selections using limiting soluble antigen, selection of high affinity clones directly from huge non-immunized repertoires etc.

Selection technique has a profound effect on the outcome, yet this pivotal factor has received little attention. An understanding of the parameters affecting panning efficiency will permit greater control over the affinity, diversity or specificity of the isolated clones leading to faster delivery of useful antibodies. The presentation will present theoretical and experimental analyses of the effects of antigen presentation, affinity, display level, clonal stability, library size etc. on the efficiency of selection. Examples of high affinity antibody isolation using biotin selection or cell surface antigens will be used to further illustrate the design of efficient selection/deselection protocols.

Production of specific human monoclonal antibodies by human lymphocytes engrafted in normal strains of mice

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Transplantation of bone marrow from SCID mice into irradiated normal mice can potentially endow the normal recipients with characteristics typical of the immunodeficient SCID mice.We were able to engraft human peripheral blood lymphocytes (PBLs) in irradiated Balb/c mice radioprotected with SCID bone marrow. Optimal engraftment was achieved with split dose of total body irradiation (4 Gy followed 3 days later by 10 Gy). Monitoring of mouse T-cells in peripheral blood indicate an inverse correlation between these cells and the engraftment of human $CD45^+$ cells in the peritoneum. After transplantation of human PBLs in such recipients, a marked engraftment of human T-cells and B-cells in the peritoneal cavity could be detected at least for 2 months, whereas significant amounts of human immunoglobulin could be detected for more than 3 months. $CD20^+$B-cells were detected in all internal organs, but were mainly concentrated in the spleen. Total human Ig in peripheral blood reached an average of 2.8 mg/ml 14 days after transplantation, and continued to be significant for several months. The use of lethal TBI followed by radioprotection with SCID bone marrow provides a general approach to achieve engraftment of human antibody producing B-cells in normal strains of mice. Such 'chimeric' mice were immunized with a variety of specific antigens such as KLH, tetanus toxoid and hepatitis B surface antigen. Specific human antibody response was detected to each antigen. We have also engrafted human PBL from anti-hepatitis B positive individuals and induced a secondary immune response by boosting with Enjerix-B (hepatitis B vaccine). Very high titers of human anti-HBs antibodies were detected in the serum compared to the titers detected prior the boosts. Splenic B-cells were harvested from these mice for raising HuMAbs by hybridoma technology. Using these 'chimeric' mice we are developing HuMAbs for cancer therapy and for viral diseases.

Single-gene-encoded novel single-chain antibodies with anti-tumor cytolytic activity

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Monoclonal antibody (MAb) CC49 reacts with the human tumor associated glycoprotein TAG-72. Radiolabelled CC49 has shown excellent tumor localization in several ongoing clinical trials. In our effort to generate potentially diagnostic and therapeutic antitumor immunological reagents, we have generated three different single-gene-encoded single-chain immunoglobulin molecules. SP2/0 murine myeloma cells transfected with each of the single-gene expression constructs expressed a single-chain protein that assembled into a dimeric molecule and was secreted into the culture medium. One DNA construct encoded a single-chain protein consisting of V_H and V_L domains covalently joined through a gly-ser linker peptide, while the carboxyl end of the V_L domain was linked to the amino terminus of the human γ1 Fc through a hinge region. The dimeric molecule of ~120 kDa was designated SCA (delta)C_LC_H1. A second single-gene construct generated a fusion protein, SCA (delta)C_LC_H1- IL-2, of ~140 kDa consisting of an interleukin-2 molecule attached to the carboxyl end of the SCA(delta)-C_LC_H1. The third single-gene construct generated a dimer (SCAcCC49) of ~160 kDa in which the carboxyl end of the constant region of the chimeric light chain and the amino terminus of the variable region of the chimeric heavy chain were joined through a gly-ser linker peptide. All three single-chain immunoglobulins competed with murine MAb CC49 for binding to TAG-72. Their cytotoxic activity was similar to that of the chimeric MAb CC49. More recently we have expressed SCA(delta)C_LC_H1 and SCA(delta)C_LC_H1-IL-2 using the baculovirus expression system. The single-gene approach makes it possible to develop an antibody producing transfectoma by single step transfection. It would also faciltate *ex vivo* transfection and *in vivo* gene inoculation of cells for gene therapy protocols.

Hybridoma rescue technology: generation of HuMAbs against respiratory syncytia virus

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Two major hurdles in developing human monoclonal antibodies (HuMAbs) as potential agents of therapeutic or diagnostic utility, are the supply of B-cells activated to produce antibodies against the antigen of interest, and the instability of human hybridomas. A combination of technologies such as *in vitro* immunization, immunization via SCID mice , and PCR amplification provides a means of overcoming these hurdles. The immunization regimes generate B-cells producing MAbs with the desired specificity. The hybridomas step produces a source of monoclonal cells from which V_H and V_L chains can be rescued via PCR amplification. Overall, this technology effectively transfers the original V-regions from the B-cell to a surrogate cell with the advantages of infinite growth, production, and specificity. We generated HuMAbs against a respiratory syncytia virus (RS virus) by combining these technologies. Human peripheral blood lymphocytes (PBLs) or spleen cells were stimulated to produce antibodies against the RS virus. The cells were fused with a human fusion partner (a number of partners were tested) to generate hybridomas producing MAbs with anti-RS virus activity. PCR was employed to rescue the V-regions which were then grafted onto the appropriate human constant region. Full MAbs were expressed in surrogate cells. A number of specific MAbs were generated by this technology.

Antigen-specific primary immune response of human B lymphocytes after *in vitro* immunization with GM3 ganglioside

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In vitro immunization of human B lymphocytes was performed with liposomes containing the monosialoganglioside GM3, with or without either complete tetanus toxoid or a synthetic T-helper epitope derived from tetanus toxin (determinant 830-843). The immunized B-cells were Epstein-Barr virus transformed and the human anti-ganglioside antibody response was evaluated using an indirect ELISA against different mono- and di-sialoganglio-sides. Clones producing antigen-specific human antibodies of the IgM isotype against the ganglio-side GM3 used as immunogen were selected and one clone, IM-11, was further characterized. In addition, a method of positive selection using GM3-coated magnetic beads has been developed which allowed us to rescue unstable clones. Studies on the specificity on the human antibodies by TLC-immunostaining with a large panel of glycolipids have demonstrated that the antobody IM-11 binds strongly to NeuAc-GM3 ganglioside, sulfated glyco lipids as well as sialylparagloboside (NeuAc-SPG) but with lower affinity. Preliminary immuno- histological staining of melanoma biopsy sections showed a selective reactivity of IM-11 with melanoma cells which varied among different tumors.

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Development of pharmaceutical formulations for therapeutic antibodies: monitoring of protein structure and stability

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The advent of recent advances in biotechnology has made possible the production of many monoclonal antibodies for use as pharmaceutical drugs with a wide range of therapeutic applications. Instrinsic to the promise of such therapies are many challenges, not the least of which is maintaining the protein's homogeneity and stability during the course of synthesis, purification, formulation and application. The development of stable dosage form for monclonal antibodies presents a major challenge to the formulation scientist. Comprehension of the underlying processes that cause heterogeneity and degradation of monoclonal antibodies require a variety of techniques and instrumentations. Systematic screening studies in preformulation and formulation are the key to development of parenteral dosage form with optimal shelf-life. As a model system for the study we have used a non-depleting IgG_4 humanized monoclonal antibody. Evaluation and optimization condition for pH, ionic strength, and temperature were carried out in order to assess the influence of various stabilizers on the antibody. Monitoring of stability was performed by conventional separation techniques, spectroscopic methods and capillary electrophoresis. The development of a pharmaceutical formulation for a therapeutic monoclonal antibody and the monitoring of its structure and stability will be discussed.