Human Antibodies 11 (2002) 29–48
IOS Press

Poster session

[Poster 1]

Architectural features of human IgG that modulate its catabolic half-life in the rat

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A series of truncated, humanised IgG1 antibodies expressed in Chinese hamster ovary cells [1] and human IgG3 monoclonal antibodies were compared to assess the influence of structural components (Fig. 1) on catabolic lifetime. The series includes L243 IgG1 (α-MHC Class II) lacking a C_H3 domain pair (ΔC_H3-IgG1), Fc with/out a hinge or carbohydrate, a single C_H2 domain, and single chain Fv fusion proteins with Fc or a hinge-C_H2 domain.

Pharmacokinetic studies in the rat gave a $t_{1/2}\beta$ of 98–104 hours for the intact IgG1 and IgG3 antibodies. The $t_{1/2}\beta$ values for ΔC_H3-IgG1 (47 hours), Fc (33 hours), the C_H2 domain (25 hours), and scFvhC_H2 (8 hours) reflect modulated catabolism of these truncated forms. These functional studies suggest that the (C_H1 + C_L), C_H2 and C_H3 constant domain pairs of IgG all contribute to longevity. Thus, no constant domain pair alone dictates the catabolic lifetime. Whilst the presence of Fc-linked carbohydrate may contribute to longevity, neither the hinge nor the variable domain pair ($V_L + V_H$) contribute significantly to increase the catabolic half-life. Comparison of IgG1 and IgG3 allotypes suggests that neither His/Arg-435 nor Tyr/Phe-436 substitutions at the C_H2-C_H3 interface within the C_H3 domain affect longevity in the rat.

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[Poster 2]

Synthesis of homogeneous neoglycoforms of IgG-Fc molecules and their functional properties

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Immunoglobulin G (IgG) is comprised of two antigen binding (Fab) regions that bind antigen, and an Fc region which can trigger a range of effector mechanisms once the antibody has complexed with antigen. Carbohydrate attached at Asn-297 within the C_H2 domain of the Fc region is required for optimal recognition of the Fc by effector ligands such as Fcγ receptors and complement. The carbohydrate consists of an array of biantennary complex forms based on a conserved pentasaccharide core ($GlcNAc_2Man_3$) with variable attachment of fucose, N-acetylglucosamine, galactose and sialic acid as outer arm residues. Since the different glycoforms can dramatically affect recognition by effector ligands it is advantageous to be able to tailor the glycoform composition.

The aim of this work is to generate biologically active homogeneous glycoforms of IgG-Fc by coupling *in vitro* a synthetic sugar to residue Asn-297 within the Fc protein. Thus, we can explore the extent to which disulphide linked sugar Fc conjugates formed *in vitro* (neoglycoconjugates) can mimic the functionality of the natural amide linked sugar Fc conjugates that are synthesised within the cell. It was established that thioaldoses can spontaneously couple to thiol groups of cysteine residues to give disulfide-linked neoglycoconjugates. A series of homogeneous thioaldoses was synthesized chemically and coupled to a truncated Fc unit engineered to have Cys-297 in place of the glycan linked Asn residue (Fc NC297). The oligosac-

ISSN 1093-2607/02/$8.00

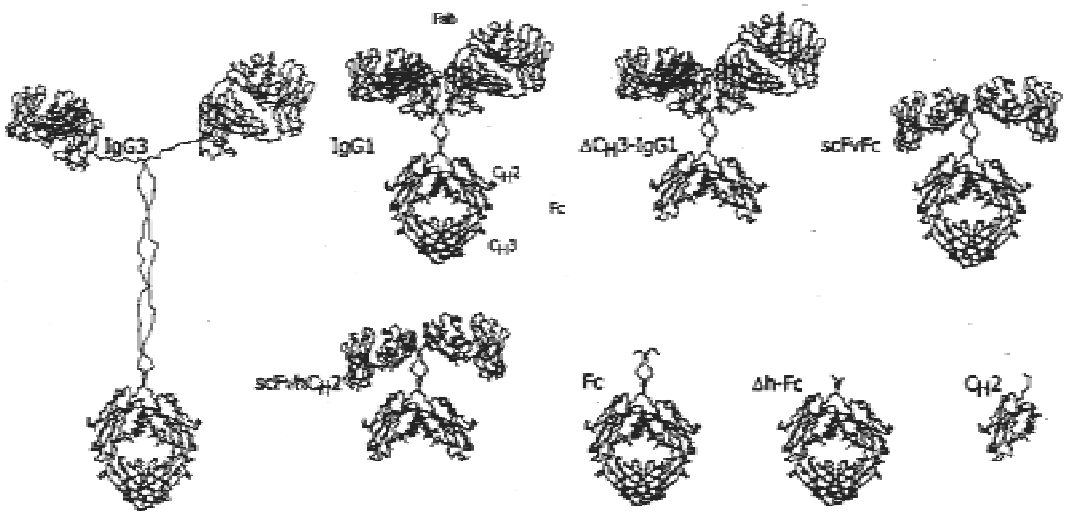

Fig. 1. Structural representations of IgG3, L243 IgG1 and its truncated forms.

charide linked to both heavy chains, as determined by MALDI-TOF spectroscopy. The series of neoglycoconjugate Fc NC297 molecules produced had one (GlcNAc), two ($GlcNAc_2$), three ($GlcNAc_2Man$), or five ($GlcNac_2Man_3$) sugar residues attached. The neoglycoconjugate Fc molecules were tested for their ability to interact with human FcγRI and thus inhibit superoxide production by U937 cells that had been stimulated with γ-interferon (Fig. 2).

Complexed L243 IgG1 was used to elicit a superoxide burst from γ-interferon stimulated U937 cells, triggered through FcγRI expressed on the surface of the U937 cells. This superoxide burst was inhibited most effectively with a recombinant Fc having native glycosylation and predominantly galactosylated oligosaccharide chains. The neoglycoconjugates showed increased inhibitory activity as the number of sugar residues of the attached carbohydrate increased, thus the rank order for inhibition of superoxide production by the FcNC297 glycoforms was 0, 1, 2, 3, 5, and 8–10 sugar residues, in ascending order of effectiveness. The resulting disulphide-linked neoglycoconjugates showed enhanced biological properties compared with aglycosylated Fc. These data suggest that the synthetic sugar molecules can substitute for the natural sugar molecules, and that disulphide-linked complex carbohydrates have the potential to mimic the natural amide linked complex carbohydrate. As the number of sugar residues comprising the introduced carbohydrate moiety increases, the resulting neoglycoconjugate becomes a more effective inhibitor. These data suggest that thioaldose sugars may have general applications to

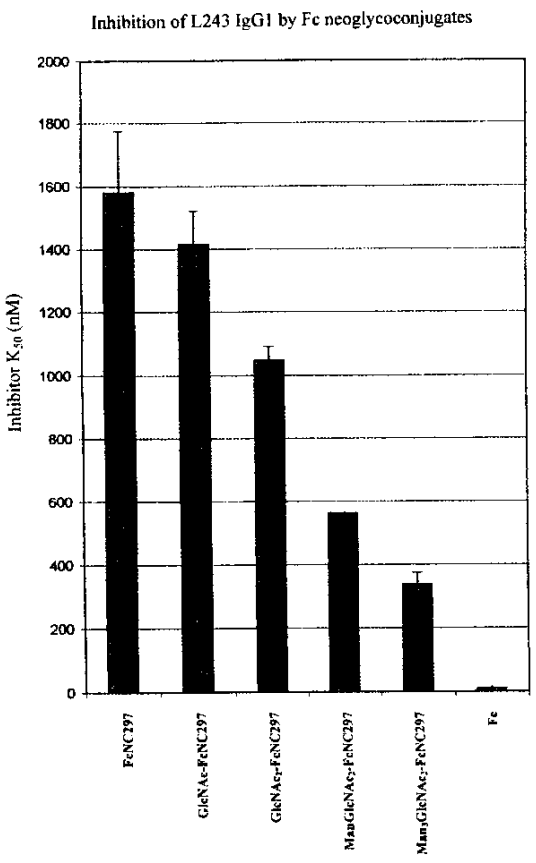

Fig. 2.

in vitro synthesis of homogeneous, functionally active glycoforms of recombinant glycoproteins.

[Poster 3]

Selective production of monoclonal antibodies based on a B cell targeting technique

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The original idea of producing monoclonal antibodies against a given antigen was described by Kohler and Milstein. However, usage of Sendai virus- or poly(ethylene glycol) (PEG)-mediated fusion methods caused non-specific fusion not only between spleen-myeloma pairs of cells, but also spleen-spleen and myeloma-myeloma pairs, resulting in lower production of the targeted hybridoma cells. Large numbers of growing hybridoma colonies need to be screened in order to find those secreting the desired monoclonal antibodies. To address this problem, we have focused on developing a new method to provide selective production of hybridoma cells secreting monoclonal antibodies with high efficiency and specificity.

A targeting method that selects antigen-specific receptors on B lymphocytes using antigen drives selective production of monoclonal antibodies against proteins and functional peptide sequences. The key step of this new method is that the receptors on antigen-immunized B lymphocytes by the antigen was confirmed by immunofluorescent analysis. This suggests that targeting B lymphocytes results in the successful and efficient production of highly specific monoclonal antibodies against not only the protein molecules, but also the lower antigenic peptide sequences. This technique could be also applicable to selective production of hybridoma cells which can specifically secrete human monoclonal antibodies.

[Poster 4]

Production and characterization of specific monoclonal antibodies of the human thyroid stimulating hormone

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Introduction: Thyroid stimulating hormone (TSH) is a pituitary glycoprotein hormone that plays a major role in the regulation of thyroid function. TSH is composed of two non-covalently linked glycosylated polypeptide chains (α and β). It is chemically and immunologically related to the pituitary and placental gonadotropins, which includes luteinizing hormone (LH), follicle stimulating hormone (FSH) and human chorionic gonadotropin hormone (hCG) [1,2].

Measurement of TSH levels in human serum requires the use of hTSH-specific antibodies in order to avoid possible cross-reaction among the related glycoprotein hormones [3].

Monoclonal antibodies (mAbs) specific for the unique structural components of the hormone have been produced, thus avoiding cross-reactivity. We have produced and characterized mAbs recognizing two epitopes specific for βTSH in order to be used in a solid phase immunoenzymatic assay.

Materials and methods: Immunization protocol: BALB/c mice were subcutaneously immunized with 50 μg of highly purified human β-TSH (SIGMA CHEMICAL, Steinheim, Germany) emulsified in complete Freund's adjuvant. Three immunizations were carried out every 21 days with the same dosage in incomplete Freund's adjuvant by intraperitoneal route. A booster of 50 μg of β-TSH in saline solution was given to the animals with the highest titer, three days before cell fusion.

Hybridization and cell culture: Spleen cells were homogenized and mixed with mouse myeloma cells (P3/X63-Ag8) at a ratio of 10:1 before fusion in polyethylene glycol 1500 (Sigma Chemical Co., St. Louis, MO, Hybrid-Max) according to Fasekas and Scheidegger [4]. The resulting cell suspension was distributed in 96-well culture plates at 1×10^5 cell/well. Hybridomas were selected in HAT medium (IMDM medium with 10% (v/v) fetal calf serum (Gibco BRL, New York, USA) supplemented by hypoxanthine 1^{-4} M, aminopterine 10^{-5} M, thymidine 3×10^{-5} M and feeders cells). After 10–15 days of culture, supernatants of the growing hybrids were screened for the presence of anti-TSH antibodies by immunoenzymatic assay. Selected hybridomas were subcloned twice by limiting dilutions; followed by large-scale production by ascitic tumors developed in pristane (2, 6, 10, 14 – tetrametyl – pentadecane) injected BALB/c mice.

Isotype determinations: The isotype of each antibody was determined by the double immunodiffusion method using antisera specific for mouse immunoglobulin classes and subclasses (Sigma Chemical CO. St. Louis, USA, MO) [5,6].

Purification of monoclonal antibodies: Immunoglobulin was purified by Protein A-Sepharose Cl-4B (Phar-

macia, Uppsala, Sweden), the IgG1 fraction was dialyzed against a phosphate-buffered saline (PBS), pH 7.2 and the aliquots were stored at – 70°C.

Specificity measurement: The specificity of monoclonal anti-TSH antibodies was evaluated with the hormones FSH, LH and hCG; assaying different concentrations of each antibody in plates coated with 1 μg/mL of each one of the hormones in 0.05 M sodium carbonate/bicarbonate buffer, pH 9.6.

Measurement of the affinity constant: The determination of the affinity constant was performed using the method described by Beatty et al. [7] with some modifications to be used in the ultramicroanalytical system. Fluorescence values obtained versus the logarithm of antibody concentration, were represented in a graph to obtain some sigmoid curves [8].

$$K_{aff} = (n-1)/2(n[Ab']_t - [Ab]_t)$$

$$\text{where } n = [Ag]_t/[Ag']$$

Determinations of the hTSH epitopes recognized by monoclonal anti-hTSH antibodies: The test for recognizing epitopes was performed by means of a competitive assay (inhibition), using ultramicroElisa plates coated with 6 μg/mL of each one of the monoclonal antibodies to TSH in 0.05 M sodium carbonate/bicarbonate buffer, pH 9.6. A solution of the antigen (TSH) was preincubated with each one of the monoclonal antibodies either 1 h at 37°C or overnight at 4°C with at least two times the molar concentration. After the preabsorption, the mixture was incubated for 2 h at 37°C in the plates coated with the MAB. Sheep anti-human TSH alpha subunit/Alkaline Phosphatase conjugate was added and the reaction was revealed using a fluorogenic substrate (4-methylumbelliferilfosfate).

Results and discussion: Hybridomas were cultured in five 96-well culture microtitration plates. Supernatants were assayed, 10–12 days after cell fusion to assess anti β-TSH antibody production. Five hybridomas ($2C_1$, $4A_2$, $5F_{12}$, $7C_{10}$, and $10G_8$), were selected for the stability, specificity and affinity for hTSH. Secretory cells of antibodies were cloned and re-cloned three times to guarantee monoclonal behavior of the produced immunoglobulines. Those immunoglobulines were characterized by double immunodiffusion method in agar gel. The fact that each mAb, produced by all the hybridomas belonged to the IgG1 subclass, was checked.

Studies went on with $2C_1$, $4A_2$ and $10G_8$ hybrid cells because they presented the highest amount of positive clones within the performed re-clon process. MAbs were analyzed, with respect to the hTSH epitope they recognized, by competitive binding assay with pairs of purified mAbs. One antibody was coated at 6 μg/mL on ultramicroELISA plates while another was used in solution (2–8 μg/mL).

Table 1
Specificities of anti-hTSH monoclonal antibodies

Monoclonal	Hormones			
Antibodies	TSH	hCG	LH	FSH
2C1-G10	+	–	–	–
4A2-A8	+	–	–	–
10G8-F4	+	–	–	–

Table 2
Affinity constant of Mabs

Monoclonal antibodies	Kaff (M^{-1})
2C1-G10	8.1×10^{10}
4A2-A8	4.3×10610
2C1-G10 + 4A2-A8	1.5×10^{11}

Competitive inhibition binding curves to hTSH between the two other mAbs and mAb 2C1-G10 or 4A2-A8 in solution are shown in Figs 1(A) and (B). According to the previous results, we can conclude that the two anti hTSH mAbs (4A2-A8 and 10G8-F4) recognize the same epitope or close epitope on the hTSh molecule and the mAb 2C1-G10 recognizes a different one. These results match with the previous studies done by other many researchers [9,10]. The studies of hTSH mapping using mAb anti-TSH revealed four distinct epitopes for hTSH, and three of them, within β chain. One of them seems to be the most immunodominant one, because 90% of the produced MAbs bind that antigenic determinant [11].

The specificity of monoclonal anti β-TSH antibodies is shown in Table 1. Evaluated antibodies have no cross-reactivity with other hormones so they are highly specific to TSH. Specificity can be explained due to the three antibodies against epitopes located in the β chain, which differs from the other hormones [12,13]. However, a possible participation of the α subunit in the conformation of the epitopes cannot be excluded, as has been suggested by Ridgway et al. [14].

Table 2 shows the results of the average affinity constant obtained in every consecutive assay that corresponds to each MAb. Therefore, monoclonal antibodies 2C1-G10 and 4A2-A8 present high affinity of binding TSH [15]. The binding of these two MAbs increase affinity which was confirmed by the obtained value of the affinity constant (1.5×10^{11} M^{-1}).

There was a selection of monoclonal antibodies (2C1-G10 and 4A2-A8) because of their high specificity and high affinity to TSH; they also recognize two different epitopes within β-TSH and they develop easy

absortion to the solid phase. They were selected to be used in coating plates of the diagnostic UMELISA TSH Neonatal kit for screening of congenital hypothyroidism in newborns. This disease is due to the anatomical or functional absence of the thyroid gland and it is the major cause of avoidable mental retardation.

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[Poster 5]

Monoclonal antibody against free β-subunit of human chorionic gonadotropin

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Introduction: Human chorionic gonadotropin (hCG) is a glycoprotein hormone composed of two non-covalently linked glycosylated polypeptide chains α (92 aminoacids) and β (145 aminoacids) [1]. The α-subunit of hCG is similar to that of pituitary and placental gonadotropins, which includes luteinizing hormone (LH), follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH) [2,3]. The β-subunit, however, is unique and distinguishes hCG from these other hormones, and antibodies have been developed to give no or minimal cross-reaction with LH, which is especially important for measuring low concentrations of hCG in the diagnosis and management of very early pregnancies, ectopic pregnancies and monitoring of neoplasm and trophoblastic diseases [4–6]. Several different molecular forms of hCG have been found during normal pregnancy in placental tissues, maternal serum, and urine. Free α and β-subunits also are detected in serum and urine [7].

Immunoassays based on the β-subunit of hCG have been employed to decrease this cross-reactivity with different molecular forms of hCG, but when these assays are used with serum specimens, the antibodies employed must recognize a new epitope on the free β-subunit which was not detect into the whole β-subunit [8–10]. To overcome these problems, we have produced and characterized a one monoclonal antibody to the free β-subunit of hCG recognizing one epitope specific for βhCG in order to be used in a solid phase immunoenzymatic assay for Down's syndrome screening.

Materials and methods: Immunization protocol: BALB/c mice were subcutaneously immunized with 30 μg of highly purified human β-hCG (SCRIPS, USA) emulsified in complete Freund's adjuvant. Three immunizations were carried out every 21 days with the same dosage in incomplete Freund's adjuvant by in-

traperitoneal route. A booster of 40 μg of β-hCG in saline solution was given to the animals with the highest titer, three days before cell fusion.

Hybridization and cell culture: Spleen cells were homogenized and mixed with mouse myeloma cells (P3/X63-Ag8) at a ratio of 10:1 before fusion in polyethylene glycol 1500 (Sigma Chemical Co., St. Louis, MO, Hybrid-Max) according to Fasekas and Scheidegger [11].

The resulting cell suspension was distributed in 96-well culture plates at 1×10^5 cell/well. Hybridomas were selected in HAT medium (DMEM/F-12 medium with 10% (v/v) fetal calf serum (Gibco BRL, New York, USA) supplemented by hypoxanthine 10^{-4} M, aminopterine 10^{-5} M, thymidine 3×10^{-5} M and feeders cells). After 10–15 days of culture, supernatants of the growing hybrids were screened for the presence of anti β-hCG antibodies by immunoenzymatic assay.

Selected hybridomas were subcloned twice by limiting dilutions; followed by large-scale production by ascitic tumors developed in pristane (2, 6, 10, 14 tetrametyl -pentadecane) injected BALB/c mice.

Isotype determinations: The isotype of each antibody was determined by the double immunodiffusion method using antisera specific for mouse immunoglobulin classes and subclasses (Sigma Chemical CO. St. Louis, USA, MO) [12,13].

Purification of monoclonal antibodies: Immunoglobulin was purified by Protein A-Sepharose Cl-4B (Pharmacia, Uppsala, Sweden), the IgG1 fraction was dialyzed against a phosphate-buffered saline (PBS), pH 7.2 and the aliquots were stored at $-70\,^{\circ}$C.

Specificity measurement: The specificity of monoclonal anti β-hCG antibodies was evaluated with the hormones LH, intact hCG and βhCG; assaying different concentrations of each antibody in plates coated with 1 μg/mL of each one of the hormones in 0.05 M sodium carbonate/bicarbonate buffer, pH 9.6.

Measurement of the affinity constant: The determination of the affinity constant was performed using the method described by Beatty et al. [14] with some modifications to be used in the ultramicroanalytical system.Fluorescence values obtained versus the logarithm of antibody concentration, were represented in a graph to obtain some sigmoid curves [15].

$$K_{aff} = (n-1)/2(n[Ab']_t - [Ab]_t)$$

$$\text{where } n = [Ag]_t/[Ag']$$

Determinations of the β-hCG epitopes recognized by monoclonal anti- β-hCG antibodies: The test for recognizing epitopes was performed by means of a competitive assay (inhibition), using ultramicroELISA plates coated with 10 μg/mL of each one of the monoclonal antibodies to β-hCG (2C5 and MAb comercial that recognize one epitope on the free β-subunit which was not detect into the whole β-subunit.) in 0.05 M sodium carbonate/bicarbonate buffer pH 9.6.

A solution of the antigen (β-hCG) was preincubated with each one of the monoclonal antibodies 1 h at 37 °C with at least two times the molar concentration. After the preabsorption, the mixture was incubated for 30 min at 37°C in the plates coated with the MAb.

Mouse anti-human hCG beta subunit/Alkaline Phosphatase conjugate was added and the reaction was revealed using a fluorogenic substrate (4-methylumbelliferilfosfate).

Results and discussion: Hybridomas were cultured in five 96-well culture microtitration plates. Supernatants were assayed, 10–12 days after cell fusion to assess anti β-hCG antibody production. Two hybridomas ($2C_5$ and $8E_9$), were selected for the stability, specificity and affinity for β-hCG. Secretory cells of antibodies were cloned and re-cloned three times to guarantee monoclonal behavior of the produced immunoglobulines. Those immunoglobulines were characterized by double immunodiffusion method in agar gel. The fact that each MAb, produced by all the hybridomas belonged to the IgG1 subclass was checked.

Studies went on with 2C5 hybrid cells because it presented the highest amount of positive clones within the performed re-clon process. MAb was analyzed, with respect to the β-hCG epitope they recognized, by competitive binding assay with pairs of purified MAbs. One antibody was coated at 10 μg/mL on ultramicroELISA plates while another was used in solution (2–10 μg/mL). It can be seen that, the MAb 2C5 in solution fully inhibited the β-hCG binding to MAb control coated on the ultramicroELISA plates. When 2C5 was coated onto the ultramicroELISA plates, the inhibition achieved by the MAb in solution exceeded 80% of the binding of β-hCG to MAb 2C5. On the other hand, when Mab control was coated onto the ultramicroELISA plates, the inhibition achieved by the Mab in solution was similar. According to the previous results, we can conclude that the two anti β-hCG MAbs (2C5 and control) recognize the same epitope on the β-hCG molecule. The studies of hCG molecule mapping using Mab anti-hCG revealed four distinct for hCG, and three of them, within β-chain. One of them seems not available in the intact hCG [3,4,10].

The specificity of monoclonal anti β-hCG antibody purified against pituitary hormones which include

luteinizing hormone (LH), intact human chorionic gonadotropin hormone (hCG) and human chorionic gonadotropin hormone subunit β (βhCG) was evaluated by means of an ultramicro-ELISA. Evaluated antibody has no cross-reactivity with other hormones so they are highly specific to βhCG. Specificity can be explained due to the antibody against epitope not available in the intact hCG located in the free β-Subunit of Human Chorionic Gonadotropin [3,4,10].

The affinity constant of this monoclonal antibody was 1.5×10^{10} M^{-1}. Therefore, Mab 2C5 presents high affinity of binding β-hCG [5,9]. There was a selection of monoclonal antibodies (2C5) because of their high specificity and high affinity to βhCG; it also recognize one epitope within β-hCG not available in the intact hCG and it develop easy absorption to the solid phase. The MAb was selected to be used in coating plates of the diagnostic UMELISA free βhCG kit for Down's syndrome screening in pregnancies. Free β-hCG in maternal serum has been shown to be increased in Down's syndrome-affected pregnancies and is proportionally increased in more cases than is total hCG [16].

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[Poster 6]

Monoclonal antibodies against hepatitis B S antigen: Production, characterization, and use for diagnosis

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Introduction: The HBV is a partially double-strained circular DNA virus of the class Hepadnaviridae. The viral particle is 42 nm in size and consists of an outer lipoprotein coat and hepatitis B surface antigen (HBsAg), which circulated in the blood in two forms: as viral particle-bound protein form or as a free, noninfectious protein presenting as 22-nm spherical and tubular particles [1]. Rapid, sensitive, and specific detection methods are indispensable for the diagnosis, monitoring, and possible prevention and eradication of Hepatitis B [2,3]. At present it is generally accepted that the most important markers to be tested in human sera are HBsAg, anti-HBsAg antibodies, HBeAg, and HBV DNA. Monoclonal antibodies recognizing definite epitopes and enabling more specific and sensitive detection

than routine polyclonal antibodies should be employed in these tests.

The aim of the present work was to prepare hybridomas producing monoclonal antibodies against HBsAg, using a recombinant and natural antigen for immunization, and develop a monoclonal for its use in the assay for detection HBsAg in human sera.

Materials and methods: Immunization and production of MAb: BALB/c mice were immunized with three IP 50 μg (Recombinan antigen or natural antigen) at 3-week intervals. Three day afte the last injection, spleen cell suspension was prepared and fused with P3/X63-Ag8 mouse myeloma cells at a ratio of 10:1 using polyethylene glycol 1500 (Serva, Paris) according to Fasekas and Scheidegger [6]. The fused cells were selected in HAT medium (IMDM medium with 10% (v/v) fetal calf serum (Gibco BRL) supplemented by hypoxanthine 10^{-4} M, aminopterine 10^{-5} M, thymidine 3×10^{-5} M and feeders cells). Subsequent cloning was prformed by the limiting dilution technique and hybridoma cells producing relevant MBAs were screened by UMELISA using Recombinan HBsAg-coated plate. The specificity of monoclonal anti-HBsAg antibody was evaluated with the HbsAg ad and ay subtypes. The immunoglobulin class was determined by the double immunodiffusion method using antiserum specific for mouse immunoglobulin classes and subclasses (Sigma Immunochemicals) [7,8].

Measurement of the affinity constant: The determination of the affinity constant was performed using some modifications of the method described by Beatty [7] to be used in the ultramicroanalytical system.

Fluorescence values obtained versus the logarithm of antibody concentration, were represented in a graph to obtain some sigmoid curves [8].

$$K_{af} = (n-1)/2(n[Ab']_t - [Ab]_t)$$
$$\text{where } n = [Ag]_t/[Ag']$$

Test for competitive inhibition: The test for recognizing epitopes was performed by means of a competitive assay (inhibition), using ultramicroELISA plates coated with 6 μg/mL of each one of the monoclonal antibodies to HBsAg in 0.05 M sodium carbonate/bicarbonate buffer, pH 9.6. A solution of the antigen (HBsAg) was preincubated with each one of the monoclonal antibodies either 1 h at 37°C or overnight at 4°C with at least two times the molar concentration. After the preabsorption, the mixture was incubated for 2 h at 37°C in the plates coated with the mAb. Sheep anti-HBsAg /Alkaline Phosphatase conjugate was added and the reaction was revealed using a fluorogenic substrate (4-methylumbelliferilfosfate).

Table 3
Competition between the various anti- HBsAg mAbs for binding to HBsAg

Coated mAbs	Inhibition (%) of HBsAg binding to coated mAbs[a]		
	2F12-H9	2C4-A2	2C4-B2
2F12-H9	73%	0%	0%
2C4-A2	0%	71%	77%
2C4-B2	0%	73%	72%

[a]Inhibition percentage of HBsAg binding to coated anti- HBsAg mAbs is evaluated when 50 μg/mL of mAbs in solution are added. Calculations are made with the maximum binding HBsAg with polyclonal antibody conjugated to phosphatase alkaline take as 100%. Assays were carried out as described in the materials and methods section and each number is the mean of duplicate determinations.

Conjugation: MAb protein concentration in milligrams per milliliter was determined from A 280 divided by the extinction coefficient, 1.38. The mAb were conjugated to phosphatase alkaline using method one step glutaraldehide [9] and biotine according to Guesdon [10].

Results and discussion: Hybridomas were cultured in five 96-well culture microtitration plates. Supernatants were assayed, 10–12 days after cell fusion to assess anti HBsAg antibody production. Five hybridomas ($2F_{12}H_9$, $2C_4A_2$, $2C_4B_2$, $3F_1F_3$, $5G_4C_2$), resulted positive. Secretory cells of antibodies were cloned and re-cloned three times to guarantee monoclonal behavior of the produced immunoglobulines. Those immunoglobulines were characterized by double immunodifussion method in agar gel. The fact that each mAb, produced by the $2F_{12}H_9$, $3F_1F_3$ and $5G_4C_2$ hybridomas belonged to the IgG_1 subclass, and $2C_4A_2$ and $2C_4B_2$ were IgG_{2b}.

The specificity of monoclonal anti HBsAg antibodies purified against HBsAg which include subtype Ad and Ay, was evaluated by means of an ultramicroELISA. Evaluated antibodies have cross-reactivity with both subtypes. Specificity can be explained due to the three antibodies recognized by the A epitopes located in the HBsAg.

Studies went on with $2F_{12}H_9$, $2C_4A_2$, and $2C_4B_2$ hybrid cells because they presented the biggest amount of positive clone within the performed re-clone process. In the test for competitive inhibition, two of the mAbs ($2C_4A_2$) and ($2C_4B_2$) recognized the same epitope, and 2F12H9 recognized a different one (Table 3).

Table 4 shows the results of the average affinity constant obtained in every consecutive assay that corresponds to each mAb. Therefore, monoclonal antibody

Table 4
Affinities of various anti- HBsAg monoclonal antibodies (mAbs) used in this study

mAb	Affinity Constant[a]
2F12H9	$3.54 \pm 1.07 \times 10^9$ M^{-1}
2C4B2	$0.685 \pm 0.68 \times 10^9$ M^{-1}
2C4A2	$0.85 \pm 0.27 \times 10^9$ M^{-1}

[a]Affinity for binding HBsAg determined by Scatchard's analysis. Each result is the mean of triplicate determinations.

$2F_{12}H_9$ presents high affinity of binding HBsAg and $2C_4A_2$ and $2C_4B_2$ present the same affinity constant.

There was a selection of monoclonal antibodies ($2F_{12}H_9$ and $2C_4A_2$) because of their high specificity and high affinity to HBsAg; they also recognize two different epitopes within HBsAg and they develop and easy conjugation to the biotine. They were selected to be conjugated of biotine at the diagnostic UMELISA HBsAg Monoclonal kit for screening of HBsAg.

The preliminary results of the evaluation of the UMELISA HBsAg Monoclonal kit employed those biotinilated mAb with a commercially available Kit (UMELISA HBsAg with detectability 0.5 ng/mL) we could determine the increment of the detectability for Ad subtype 0.12 ng/mL and Ay subtype 0.15 ng/mL with reference to standard Paul Ehrlich Institute, Frankfurt, F.R.G. The detection limit is similar to other third-generation assay (0.05–0.2 ng/mL), with a high specificity, characteristic of ELISAs based entirely on mAbs.

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[Poster 7]

Specific monoclonal antibody against the human trypsin

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Introduction: In the human pancreas, trypsin is one of the pancreatic enzymes produced. Thus, its level in blood is a specific marker for pancreatic function [1]. Trypsin is synthesized and secreted from the acinar cells of the pancreas as enzimatically inactive trypsinogen (MW24000-28000). Two distinct trypsinogen isoenzymes have been isolated: cationic trypsin (trypsin-1), and anionic trypsin (trypsin-2). Since the discovery by Crossley et al. [2] of a high concentration of trypsin in serum of newborns with cystic fibrosis, many trypsin monoclonal antibodies have been developed using different antigen preparations of human trypsin-1 [3,4]. The molecular species that are increased in cystic fibrosis blood-spots are probably zymogens of trypsin [3,5]. For purification Trypsin(ogen), standard preparation and immunization we suggest the development production and characterization of monoclonal antibodies (mABs) against trypsin-1.

Materials and methods: Immunization and production of Mab: BALB/c mice were immunized with three IP 50 μg of Trypsin-1 Phenylmethylsulfonylfluoride (PMSF) at 3-week intervals. Three day afte the last injection, spleen cell suspension was prepared and fused with P3/X63-Ag8 mouse myeloma cells at a ratio of 10:1 using polyethylene glycol 1500 (Serva, Paris) according to Fasekas and Scheidegger [6]. The fused cells were selected in HAT medium (IMDM medium with 10% (v/v) fetal calf serum (Gibco BRL) supplemented by hypoxanthine 10^{-4} M, aminopterine 10^{-5} M, thymidine 3×10^{-5} M and feeders cells). Subsequent cloning was performed by the limiting dilution technique and hybridoma cells producing relevant MBAs were screened by UMELISA using Trypsin-coated plate. The specificity of monoclonal anti-Trypsin-1 antibody was evaluated with the cross-reactant enzymes-coated plates. The immunoglobulin class was determined by the double immunodiffusion method using

antiserum specific for mouse immunoglobulin classes and subclasses (Sigma Immunochemicals) [7,8]. Purification of monoclonal antibodies: The ascitic fluid was collected ten days later and centrifuged at 12 070 $\times$ g at 4°C. Immunoglobulin was purified by Protein A-Sepharose CL-4B (Pharmacia), the Igs fraction was dialized against a phosphate-buffered saline (PBS), pH 7.2 and the aliquots were stored at -70°C.

Measurement of the affinity constant: The determination of the affinity constant was performed using some modifications of the method described by Beatly et al. [9] to be used in the ultramicroanalytical system [10]. Fluorescence values obtained versus the logarithm of antibody concentration, were represented in a graph to obtain some sigmoid curves [11].

$$K_{af} = (n-1)/2(n[Ab']_t - [Ab]_t)$$

$$\text{where } n = [Ag]_t/[Ag']$$

Purification of trypsinogen and trypsin: Human pancreatic acetone powder was extracted by the procedures previously described [12]. Trypsinogen was purified by immunoaffinity, employing the Sepharose-Monoclonal Antibody (3H9), which was prepared by coupling at pH 6.5 to cyanogen bromide activated (10–12 mg/mL of AcM 3H9) with 98.5% of coupling. After coupling mAb-Sepharose conjugate was washed with 10 mM NaCl. For elution 0.2 M of glycine pH 2.8 was employed.

Results and discussion: Hybridomas were distributed into 960 wells containing HAT, and after screening, 5 wells brought about a positive result. Several hybridomas, which initially secreted antibodies, ceased to secrete or died after subsequent culture or subcloning. Only one hybridoma (3H9) was successfully established and seemed stable.

The production of monoclonal Trypsin-1 antibodies from 3H9 hybridoma was carried out "*in vivo*" using BALB/c mice, and the ascitic fluid was purified by Protein A-Sepharose CL-4B.

Immunoglobulin class determination showed mAb 3H9 to be IgG1 class and. not react with any other enzyme than human tripsin(ogen). When samples were treated with β-mercaptoetanol, immunodetection was observed on reduced proteins with mAb. This result indicates that the monoclonal antibody is directed against sequential epitope [13,14].

Monoclonal antibody 3H9 which presents high specificity and affinity to Trypsin-1; was selected to develop one-step affinity chromatography purification of Trypsin-1. The elution profiles the Sepharose-mAb indicated one protein peak, which represents absorbed material (Fig. 3). One protein component was eluted and was found to be cationic trypsinonen-1 (inactive form). Purified enzyme was lyophilisating-giving trypsinogen-1.

Table 5
Affinity constant employed as antigen Trypsin-1 from SCRIPPS and purified Trypsinogen

Antigen	Affinity
SCRIPPS	1.79×10^9 L/M
Purified Trypsinogen	1.89×10^9 L/M

The affinity constant obtained with the purified zymogen was similar to the commercial trypsin-1 (SCRIPPS) (Table 5). This result supports the conclusion that the purified enzyme is trypsinogen. We have taken advantage of the properties of monoclonal antibody to produce an affinity column for the purification of human proteins. This is the first report of the employed monoclonal antibody in trypsinogen purification form pancreatic extract. The trypsinogen obtained was useful for the preparation of standard material for immunoassay and for immunization of mice, it was also useful in the development of another monoclonal antibody to design immunoassay to trypsin quantification.

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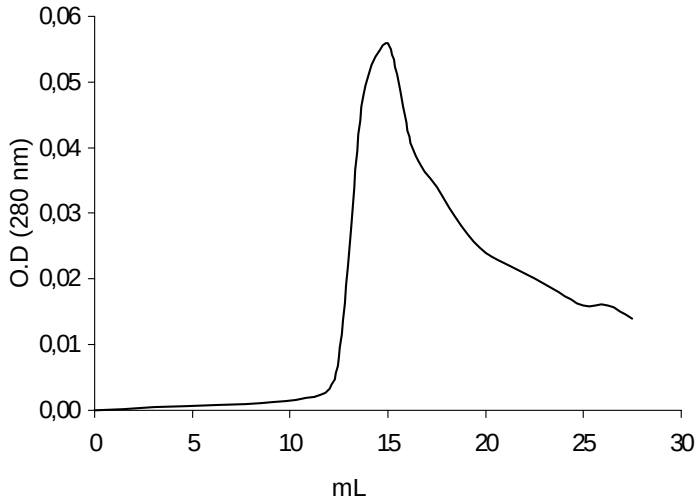

Fig. 3. Chromatography of pancreatic extracts on Sepharose-mAb.

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[Poster 8]

Human miniantibodies against ebola virus

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Ten clones, producing phage antibodies specific to Ebola virus, have been selected from a combinatorial phage library of human single-chain antibody fragments, scFv, (Medical Research Council Centre, Cambridge, England) using biopanning procedure with inactivated Ebola Zaire virus. Then selected antibodies were obtained as individual molecules of soluble miniantibodies. These human monoclonal miniantibodies were assayed for their binding with inactivated and native Ebola virus using ELISA, and it was shown that from ten miniantibodies specific to inactivated Ebola virus only nine miniantibodies were able to bind active virus. Specificity of the miniantibodies was assayed using immunoblot analysis; six miniantibodies bound NP, VP40, VP35 or VP24 of Ebola virus while four miniantibodies did not bind Ebola proteins fractionated electrophoretically. Testing of selected antibodies in binding reaction with inactivated Marburg virus revealed crossreactivity for a half of the miniantibodies. In addition, neutralizing activity for the selected miniantibodies was assayed in plaque reduction neutralization test using VeroE6 monolayer.

[Poster 9]

Generalization of a new method to reduce immunogenicity of chimeric monoclonal antibodies

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Several approaches have been developed to reduce the human immune response to non-human antibodies. However, chimeric antibodies and humanized antibodies often have decreased binding affinity. We described a new approach for reducing the immunogenicity of chimeric antibodies while maintaining the affinity. This approach seeks to prevent the recognition of murine immunogenic peptides from the antibody variable region by human lymphocytes. Putative immunogenic epitopes in the variable region are identified and subjected to site directed mutagenesis to make them human and/or to break the amphipatic motifs. The R3 and T1 antibodies, which blocks the EGF receptor, and recognized the human T cell surface molecule, CD6,

respectively were used as a model systems to test this approach. For R3 antibody four segments containing possible amphipatic epitopes were found in the heavy variable domain using the program AMPHI. Six amino acids within two of these segments were substituted by the corresponding residues from a homologous human sequence. No mutations were made in the murine light variable domain. For T1. a long segment containing possible amphipatic epitopes were found in the light chain, and seven changes were proposed substituted by the corresponding residues from a human homologous sequence. In the heavy chain, three amphipatic segments were found and four changes were done. Experiments in monkeys suggested that the "detope" R3 and T1 antibodies were less immunogenic than its chimeric analog. A search for possible amphipatic epitopes in the Kabat database revealed the presence of conserved patterns in the different families of variable region sequences, suggesting that the proposed method may be of general applicability.

[Poster 10]
Detection of schistosoma haematobium antigens in serum and urinary tract tissue of infected hamsters using specific monoclonal antibody
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This study aimed to assess the diagnostic potential of anti- Schistosoma haematobium (S. haematobium) soluable egg antigen (SEA) monoclonal antibody (Mab) (2F/11F) at different life cycle stages of the parasite. Furthermore, the relation of serum and immunofuorescent expression of S. haemotobium antigens in the urinary tract tissue of infected hamsters to histological changes was evaluated during the course of S. haematobium infection (6 months). This Mab detected antigenic epitopes expressed in all S. haematobium developmental stages. Schistosome antigens both in serum and tissue, were detected from the first month of infection and preceded any pathological changes. From the second to fourth month post-infection (p.i.), the level of schistosome antigens in the serum as well as its expression in the tissue were increased parallel to the pathological changes in urinary tract tissue. Circulating antigen level gradually decreased starting from the fifith month p.i. However, no significant changes were detected in the tissue expression of S. haematobium antigens and pathological changes at 5th and 6th month p.i. compared to 4th month. These findings suggest that the detection of schistosome antigens in serum and/or tissue can be used asa predictive marker of pathological changes in the urinary tract od S. haematobium infected hamsters.

[Poster 11]
Assessment of monoclonal antibodies for the diagnosis of schistosoma haematobium infection
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Detection of circulating schistosomiasis antigens might prove to be a good quantitative test which correlates with intensity of infection.

In the present study the kinetics of circulating schistosome antigens (CSA) in sera and tissue of Schistosoma haematobium soluble egg antigen monoclonal antibodies (Mabs) of diagnostic potential were used. From a panel of Mabs, two IgG1 Mabs (2D/11C and 10B/2C) were strongly reactive with antigenic epitopes on S. haematobium and non-reactive with either S. mansoni or other parasite antigens. The dynamics of serum and tissue schistosome antigens were studied in relation to pathological changes in the urinary tract of S. haematobium infected hamsters during the course of infection (24 weeks). CSA was detected in infected hamsters sera 3 weeks post-infection (p.i.), reaching its peak 10 weeks p.i. and remained at a low but detectable level till the end of the infection. These two Mabs were further used for measuring CSA levels in sera of 65 S. haematobium-infected patients. Serum samples from 25 S. mansoni-infected patients, 15 patients harboring other parasites, and 15 non-infected individuals were also assessed. CSA was detected in 89% of sera from S. haematobium infected patients. While, CSA was undetectable in all sera from S. mansoni-infected patients, healthy subjects and patients with other parasitic infestations.

Our data show that the use of anti-S. haematoium Mabs provides a highly sensitive and specific method for early diagnosis of Schistosomiasis haematobium (starting at 3rd week p.i.). The present work also recommends the use of Mabs as predictors for histopathological changes.

[Poster 12]
TNF related apoptosis inducing ligand (TRAIL) receptor antibody agonists
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We have developed a program that interfaces cell-based screening with hybridoma production to isolate functional antibody agonists and antagonists. Our initial efforts have focused on the TNF receptor superfamily, an important class of immunomodulatory transmembrane proteins. TNF-related apoptosis-inducing ligand (TRAIL, Apo2L) is a member of the TNF cytokine family. Upon binding to DR4 or DR5, two members of the TNF receptor superfamily, TRAIL induces cell death by apoptosis. *In vitro*, TRAIL has been shown to kill tumor cells but is relatively non-toxic to normal cells. This result has been verified *in vivo* using a soluble active recombinant form of the ligand. Studies using mice and non-human primates have demonstrated that TRAIL induces tumor regression while having little or no effect on surrounding normal tissue. Although TRAIL is emerging as a potential cancer therapeutic, little is known about the mechanisms underlying the specificity of TRAIL for malignant cells. This is primarily due to the fact that TRAIL has been shown to specifically interact with five different TNF receptors. We have isolated several anti-human DR4 and DR5 antibody agonists that mimic the action of the ligand. These specific antibodies induce apoptosis in tumor cells and have little effect on normal cells *in vitro*. Having specific TRAIL receptor antibody agonists should provide a means to decipher the mechanisms underlying tumor specificity. In addition, these antibodies may represent potential specific therapeutic agents for the treatment of cancer.

[Poster 13]

Production of various human monoclonal antibodies for medical, biological and agricultural research

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Human monoclonal antibodies produced by human-human hybridomas were useful for diagnosis and therapy of various diseases. However, there are many problems with their practical uses as medicines. We would like to develop human monoclonal antibodies not as medicines but as research reagents, and try to supply them to laboratory scientists. The human fusion partner cell line, SK-729-1, was obtained from burkitt lymphoma cell. This partner cell line has the hybridomas generating high antibody productivity. When this SK-729-1 was used for cell fusion, the amounts of monoclonal antibodies were 0.9 pg/cell /day on average. This value corresponds to five times than that of general hybridomas reported by any laboratories. This results in a reduction in the price os the antibody reagents. Fusion partner cell SK-729-1 can grow in serum free media consisting of insulin, transferring ethanolamine, and sodium selenite. Since hybridomas derived from SK-729-1 could also proliferate in serum-free media, purification of the supernatant containing monoclonal antibodies was effective without contamination of the serum proteins. We obtained 9 kinds of human monoclonal antibodies: antibodies reactive to carcino embryonic antigen (CEA), prostate specific antigen (PSA), human gonadotropin (hCG), DerFI, DerFII antigens from Dermatophagoides farinae, hepatitis B virus surface antigens (subclass -Cad, -ay). By analysis of the fluorescence intensities data from a flow cytometer and fluorescence microscopy, antibody specificity and quantity were rapidly measured. It was found that the cells must be kept at 4 degrees centigrade to obtain a high contrast image without non-specific fluorescent radiation. A stock tube for preserving monoclonal antibodies was selected to use in a marketing by a freeze-thawing test. Furthermore, it was clear that the tube must be kept below 20 degrees centigrade in order to maintain the antibody activity.

[Poster 14]

Human single chain Fv antibody specific to human monocyte chemoattractant protein-1 (MCP-1) with the inhibitory activity on the monocyte chemotaxis

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MCP-1 is involved in the development of asthma, glomerulonephritis, rheumatoid arthritis, atherosclerosis, inflammatory bowel disease, and meningitis as well as in the accumulation of macrophage in tumor sites. Therefore, human anti-MCP-1 antibody may be useful for the therapeutic treatment of these diseases.

In this study, the human single chain Fv antibody library (Vγ-Vκ, Vγ-Vλ, Vμ-Vκ or Vμ-Vλ ligated into the pCANTAB 5E phagemid vector) was prepared from of the peripheral blood mononuclear cells of 20 healthy subjects as described by J.D. Marks et al. (J. Mol. Biol., 222:581, 1991). The phage display library was panned with the MCP-1-coated plastic plate and the binding specificity was confirmed by ELISA and BIAcore.

From the antibody library (Vγ-Vλ), we isolated five phage clones with the MCP-1-specific binding activity. We tested the influence of the soluble scFvs purified from these phage clones on the chemotaxis of THP-1 cells and human peripheral monocytes. One out of these clones inhibited the chemotactic activity of human MCP-1 *in vitro*.

[Poster 15]

Human single chain Fv (scFv) antibody specific to human IL-6 with the inhibitory activity to IL-6-signaling

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Human anti-IL-6 antibody may be useful for the immunotherapy of various inflammatory diseases such as rheumatoide arthritis. As the IL-6 is a growth factor for the B cell hybrydoma, it is uneasy to isolate murine B cell hybridomas producing the anti-IL-6 antibody with the IL-6-signaling inhibitory activity. In this study, The antibody library (Vγ-Vκ, Vγ-Vλ, Vμ-Vκ or Vμ-Vλ ligated into the pCANTAB 5E phagemid vector) was prepared from of the peripheral blood mononuclear cells of 20 healthy subjects as described by J.D. Marks et al. (J. Mol. Biol., 222:581, 1991). The phage display library was panned with the IL-6-coated plastic plate and the binding specificity was confirmed by ELISA and BIAcore.

From the antibody library (Vγ-Vλ), we isolated five IL-6-specific phage clones. We tested the effects of the soluble scFvs purified from these phage clones on the growth of IL-6-dependent human cell, KT-3. Two out of these clones significantly inhibited the growth of KT-3 while three showed no inhibition.

[Poster 16]

Human FcεRIα-specific human single chain Fv (scFv) antibody with the inhibitory activity on the histamine release assay

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The α-chain of FcεRI plays a critical role in the binding of IgE to FcεRI. Fully human antibody interfering this interaction may be useful for the prevention of IgE-mediated allergic diseases. Here, we describe the successful isolation of a human single-chain Fv antibody specific to human FcεRIα using the human antibody phage display libraries. We used the non-immune phage antibody libraries which were constructed from the peripheral blood lymphocytes cDNA from 20 healthy subjects. We isolated two phage clones (designated FcRε51 and FcRε70 phages) after two rounds of the biopanning selection. The purified soluble scFv, FcRε51 inhibited the binding of IgE to recombinant FcεRIα, although both FcRε51 and FcRε70 showed the fine binding specificity to FcRεI. As the FcRε51 was elucidated as a monomer by HPLC, the affinity of FcRε51 to FcεRIα was estimated by Biacore to be Kd $= 8.5$ nM that is ten-fold lower than that of IgE binding to FcεRIα. With this characteristics, the

FcRε51 exhibited the inhibitory activity on the release of histamine from passively sensitized human peripheral blood mononuclear cells.

[Poster 17]
Development of novel antigen binders based on dimerisation of VH domains
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Immunoglobulins have a wide range of recognition specificities, useful in a variety of fields, from research to diagnosis and treatment of diseases. Following the development of monoclonal antibodies, new technologies have been introduced to increase the source of specific binding molecules, most of them based on immunoglobulins' VL-VH scaffold.

Although typically the hypervariable loops (CDRs) of both light and heavy chain V regions contribute to the formation of the binding site, heavy chains have a larger significant binding ability than light chains. Sequence diversity in antigen receptor is not evenly distributed among all six hypervariable loops, but highly concentrated in the CDR3 of VH. This variability appears both in the length and sequence, and therefore shape of the CDR3 loop.

Here we present data that demonstrate that VHs are efficiently expressed in bacteria and mammalian cells as dimers with specific binding activities, indicating that VH dimers (VHD) constitute a novel scaffold for antigen recognition. The very large diversity of VH domains in terms of sequence variations, as well as the higher number of residue contact interactions that generally participate in antigen recognition, makes these domains ideal candidates to improve binding properties.

The hydrophobic patch that contributes to the interaction between VL and VH is highly conserved. In the VH, it comprises a large number of residues (between 16 and 22) located mainly in FR2 and FR4. We demonstrated that in VHDs, the two VH interact and are oriented in a similar way to VL/VH pairs forming a relatively stable association.

Using phage display technology we selected specific hetero- and homo-dimeric binders, demonstrating that also symmetrical surfaces can be formed. In addition, we show the involvement of both VH domains in antigen recognition. A glutenin specific homo-VHD binder (VHDH16) showed high affinity and specificity in ELISA and western blots. Constructs with VHH16 paired with non-related VHs completely abolished recognition of glutenin.

VHD were efficiently expressed retaining their binding specificity both, in prokaryotic and eukaryotic expression systems, in different formats including single chain (scVHD), double chain (dcVHD), Fab (Fab-VHD) and whole antibody (IgVHD) molecules.

[Poster 18]
Recombinant antibodies in cancer treatment
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The incidence and mortality of cancer has increased in the last years, moving our efforts to develop new diagnostic and treatment approaches. In that way, several monoclonal antibodies (mAb) have been used as potential therapeutic agents, using alone or in combination with radioisotopes, drugs, cytoquines or other proteins. However the use of murine-derived mAb has clinically been limited because the repeated administration of foreign immunoglobuline elicits an inherent immune response in patients. Genetic engineering has facilitated the production of molecules less immunogenic with enhanced effectors functions. We focused our attention to produce recombinant antibodies against tumors antigens in mama and colon cancer.

Ior-C5 is a murine IgG1 monoclonal antibody. It has showed a highly specificity for a novel colorectal antigen, a glycoprotein carbohydrate chain preferentially expressed on the surface of malignant colorectal cells. Immunocytochemical staining has showed a strong surface and cytoplasmatic reactivity with colorectal cancer cell lines, but not with other cell lines. This mAb has undergone preclinical analysis, toxicological and localization studies. Tumor targeting phase I/II studies in colorectal cancer patients have shown a high sensibility and well defined images few hours after administration, even in metastases.

The 14F7 mAb is a mouse IgG1 that recognizes N-glycolyl-GM3 with high specificity. In an immunohistochemical study carried out on fresh tissue sections of human benign and malignant tumors, the 14F7 mAb reacted with ductal infiltrating breast carcinoma and melanoma tissues. In experiments carried out *in vitro*, 14F7 was capable of killing cells bearing the N-glycolyl-GM3 via complement-dependent cytotoxicity. Furthermore, in experiments carried out in mice the 14F7 mAb showed a remarkable inhibition of the growth of solid tumors.

In the present study we constructed chimeric and humanized versions of these antibodies, which retained similar reactivity as the murine antibodies. Currently we are carrying out a study in mice with ior-C5 mAb to analyze his antitumoral properties, and a clinical assay in human to prove the immunodiagnosis properties of 14F7 mAb.

[Poster 19]
Classification of Rift valley fever virus strains using monoclonal antibodies
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Rift valley fever virus (RVFV), member of the a phlebovirus genus of the bunyavirideae family, is an arthropod-borne virus which emerges periodically throughout Africa where it is a major cause of endemic and epidemic disease of human and domestic livestock. Several monoclonal antibodies were prepared against the Phlebovirus Rift valley fever strain ArB1976 (Zinga). All of these were specific for the major envelope protein ie the glycoproteins G1 and G2. The monoclonal antibodies were tested by indirect immunofluorescence against a panel of reprsentative strains of RVFV, collected over a period of 38 years and isolated from various hosts in different countries. The strains were group in three distinct lineages: West African, East-Central African and Egyptian lineages. The monoclonal antibodies reacted with all of the East-Central African and Egyptian strains examined but none of the virus belonging to the West African lineage. Experiments with more strains from the different lineages are ongoing to confirm these results.

[Poster 20]
Immunoscintigraphy of experimental tumor models using 99mtc labelled monoclonal antibodies
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Breast cancer is the second most common malignancy among South Indian female population. Diagnosis of premalignant lesions and early stage primary tumors is crucial for the success of cancer therapy and increased survival rates. Despite several advances made in the field of diagnosis such as CT Scan, ultrasound, Mammogram, MRI etc., yet many small occult lesions are not diagnosed at an early stage.The members of type I growth factor receptor family, particularly EGFR and C-erbB-2 are overexpressed in a wide range of tumors. They therefore offer themselves as targets for diagnosis and therapy with monoclonal antibodies raised against their extracellular domains. Immunoscintigrapghy using 99mTc labelled monoclonal antibodies is a versatile technique for early invivo detection and localization of any primary recurrence or micrometastasis. With this aim, a panel of MAbs have been generated against EGFR and C-erbB-2 and their diagnostic potentiality in localizing the small malignant tumors was studied in experimental tumor models with overexpression of EGFR and C-erbB-2, using 99mTc labelled monoclonal antibodies. Monoclonal antibodies were reduced by the method of Mather and Ellison [1] and labelled with 99mTc using Amerscan MDP kit. After assuring the labelling efficacy to be $>$90%, required amount of 99mTc labelled MAb was injected i.v through the tail vein and scan pictures taken at regular intervals of 10 min, 2h, 6h, 12h and 24h. At the end of 24h scan, the animals were sacrificed and Biodistribution studies performed. The results of Biodistribution studies were compared with the Scan pictures and both were in good correlation, confirming that the antibody has been localised only to the regions where there is malignancy.

In conclusion, our results show that Radioimmunoscintigraphy with 99mTc labelled monoclonal antibodies might serve as a potential diagnostic tool in the field of Oncology.

Reference

[1] S.J. Mather and D. Ellison, Reduction-Mediated Technicium-99m labelling of Monoclonal antibodies, *J. Nucl. Med* **31** (1990), 692–697.

[Poster 21]
***In vivo* diagnostic and therapeutic applications of mab cibcnsh3 generated to the human epidermal growth factor receptor**
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Members of type-1 growth factor receptors especially EGFR and C-erbB-2 have been found to be overexpressed in a wide range of tumors. They therefore offer themselves as target for diagnosis and therapy of cancer. Overexpression of EGFR has been reported in several human cancers like breast, ovary, head and

neck, etc. and is often associated with poor prognosis. Some of the MAbs directed to the extra-cellular domain (ECD) of EGFR are found to have diagnostic and therapeutic application. The MAb CIBCNSH3 generated in this laboratory against the human EGFR has been evaluated for its *in vivo* diagnostic potential by performing Immunoscintigraphic studies by ^{99m}Tc SPECT analysis using nude mice xenograft with over-expression of EGFR. *In vitro* studies by ligand binding assay have revealed the anti-tumor activity of this MAb with its ability to inhibit tumor cell proliferation. The *in vivo* therapeutic efficacy has been studied using animal tumor models and compared with that of conventional chemotherapeutic agents. The results of these studies are presented in this paper.

[Poster 22]

Intracellular immunization with different anti-HIV-1 human antibodies

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The gene therapeutic approach involving intracellular immunization has been used extensively *in vitro* to inhibit human immunodeficiency virus type 1 (HIV-1) replication by using different genes coding for antibodies against regulatory viral proteins (rev, tat) enzymatic viral proteins (integrase and reverse transcriptase) or structural viral proteins (p17 and env).

A human monoclonal antibody against a conserved part of gp41 obtained after immortalization with EBV of B cells of an asymptomatic infected individual was used to construct a plasmid containing the cDNA of the single-chain variable fragment corresponding to this antibody (scFvgp41). After a stable transfection of this scFv in HOS cells expressing CD4 and CCR5 receptors a complete inhibition of mature virions was observed.

As protein transduction with several cell-penetrating peptides (CPP) is possible and permit the direct delivery of the gene product itself we used different CPP (Tat peptide, Chariot peptide or antennapedia peptide) to introduce human anti-HIV-1 antibodies (anti-env and tat) inside cells infected or not with HIV-1. The different biological activities of these human monoclonal antibodies on the replication of HIV-1 will be presented in different cell lines.

[Poster 23]

Do B-cell lymphomas arising in patient with type II cryoglobulinemia sustain the production of the cryoprecipitable IgM?

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HCV causes chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, but it is also the leading cause of type II mixed cryoglobulinemia (MC), a systemic immune complex-mediated disorder characterized by monoclonal B-cell proliferation. Because MC evolves frequently into B-cell non-Hodgkin lymphoma (NHL), chronic HCV infection has been also proposed as an etiologic factor for a subset of B-cell lymphoma.

Various studies suggest an indirect role of the virus in the pathologic process of lymphoproliferation. Futhermore, both heavy and light chain complementarity-determining regions (CDRs) of IgR from HCV-associated NHLs showed the highest similarity to antibodies with RF activity that have been found in the MC syndrome, thus suggesting that a common antigenic stimulus is involved in both MC syndrome and HCV-associated NHLs.

Nevertheless, yet it has not been proven that immunoglobulins produced from B cell NHLs in HCV positive patients are responsible of the clinical manifestations of the MC. Neither if the tumor is originated by a clone at risk because overstimulated in MC which produce proteins with characteristics of cryoglobulinemia.

A selected patient (61 year, man), with a previous open HCV-associated MC history and a lymphoplasmacytoid lymphoma of recent onset was studied.

Nucleotidic sequences of the immunoglobulin variable regions genes present in the lymphoma has been define and the most similar VH and VK regions to those germline was performed by sequence comparison. Sequencing data showed that the assigned germline counterparts of the VH genes present in the lymphoma biopsy specimen were closely related to IGHV1-2*02, IGHD2-15*01, IGHJ4*03.

At the same time, cryoprecipitate from fresh cell-free plasma was obtained. Monoclonal IgM was then purified from cryoprecipitate by gel filtration fractionation, followed by an IgM antibody-affinity chromatography.

Variable regions of antibody heavy and light chains of IgM-Fab fragments, were generate by trypsin digestion and then separated by SDS-polyacrylamide gel electrophoresis. VH and VL proteins were identified by Matrix-assisted laser desorption/ionization-mass spectrometry, following in-gel digestion with trypsin, and matching with the theoretical peptide mass of known proteins deduced from NHL-immunoglobulin sequences. At currently peptide mass fingerprinting is partially kwon and preliminary data show a compatibility between the two sequences.

[Poster 24]

The analysis of immunoglobulin variable regions of monoclonal B-cell expansions in Sjögren's Syndrome reveals similarities with HCV-associated lymphoproliferations of Type II Cryoglobulinemia

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Sjögren's syndrome (SS) and type II cryoglobulinemia (MC) are autoimmune and lymphoproliferative disorders characterised by B-cell oligoclonal or monoclonal expansion. While a number of evidences have demonstrated the association between type II cryoglobulinemia and HCV infection, the etiologic factor of SS is still unknown, although HCV and other sialotropic viruses have been implicated in some cases.

Both diseases share some characteristics: a generally indolent clinical course, production of autoantibodies, and cryoglobulinemia. Some patients develop a malignant B-cell lymphoma, predominantly of low grade, with localisation in organs site of autoimmune disease in patients with SS or site of chronic HCV infection in patients with MC. The presence of common idiotypic determinants expressed by the pathologic B-cell clones expanded in the two diseases further suggests the existence of a link between SS and type II cryoglobulinemia.

To better understand the pathogenetic mechanisms involved in lymphoproliferative processes in these two diseases, we analysed the variable heavy chain (VH) and light chain (VL) sequences of the B-cell receptor, somatic hypermutation and intraclonal variability of monoclonal B-cell expansions from LESA (Lympho-Epithelial Sialo- Adenitis) in 7 patients with SS, two of which evolved to malignant lymphoma during the follow-up.

Sequence analysis disclosed a biased use of Ig genes both for heavy and light chain (V1-69, V3-7 and V4-59 for the heavy chain and KV3-20 or KV3-15 for the light chain), evidence of selection against nonconservative mutations in FR regions, a restricted length and similar aminoacidic motifs in the CDR3 regions, suggesting a common antigen-binding specificity. Moreover, evidence of ongoing mutation and selection was obtained from the longitudinal analysis of a lymphoproliferative lesion evolved to MALT lymphoma in a patient with SS.

The peculiar molecular features displayed by lymphoproliferations in SS patients share several similarities to MC-associated premalignant B-cell expansions as well as to HCV-associated lymphomas, thus suggesting that common mechanisms of stimulation or selection, possibly due cross-reactivity or molecular mimicry, could be involved in the evolution of these diseases.

[Poster 25]

Efficient selection of high affinity phage antibodies against haptens using cleavable biotin-labeled haptenic derivative

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Creating a vast library of genetically-engineered antibodies combined with the phage display system offers a potential for creating novel antibody species that equip much improved affinity and/or specificity to a variety of antigens. Such ultrahigh affinity antibodies as to show the Ka values exceeding 10^{12} M^{-1} should enable immunoassay systems with a subfemtomole range sensitivity even based on the competitive principle, and thus should be particularly useful for trace characterization of small molecules (haptens). However, successful data for generating the artificial antibodies with the ultrahigh affinity have seldom been reported so far. It is conceivable that such high affinity phage antibodies can hardly be isolated by usual biopanning using immobilized antigens, because of the difficulty in dissociating very strong antigen-antibody interaction.

From these points of view, we developed a new selection method using the haptenic derivative directly labeled with a "cleavable biotin (CB)" as a key reagent. In this study we selected 11-deoxycortisol (11-DC),

a corticosteroid, as a model hapten. A CB-labeled 11-DC was synthesized by coupling an 11-DC derivative having a substituent [-S$(CH_2)_2$CONH$(CH_2)_2NH_2$] at the 4-position with a commercially available CB-labeling reagent having the linker involving a disulfide (SS) bond and a carboxylic acid N-succinimidyl ester group. This labeled compound showed high reactivity both with a mouse anti-11-DC antibody and with an avidin simultaneously, and its SS bond could easily be cleaved by the treatment with 50 mM dithiothreitol (DTT). To examine the efficiency of the selection, we added the CB-labeled hapten to a mixture of the phage displaying the single-chain Fv fragment (scFv) showing high affinity to 11-DC (Ka 1.3×10^{10} M^{-1}) and the phage with the scFv against (1R)-bufuralol (ratio, 1:100000), and then the biotin moiety was captured on NeutrAvidin-immobilized immunotube. After washing for removing nonspecific phages, the bound phage was recovered by the DTT treatment, infected *E. coli* XL1-Blue cells, phage rescue was performed, and the resulting phage was submitted to next cycle of the selection. Five cycles of the procedure allowed a successful isolation and enrichment of the 11-DC-specific phage out from 100000-fold excess nonspecific phage particles (specific phage $>$90%; examined by a colony PCR).

Because of the independency of the antigen-antibody affinity for recovering the bound phage antibody, the present method will be a universal procedure for generating the engineered antibodies against various antigens showing ultrahigh affinity, which will be useful not only for *in vitro* applications such as immunoassays but also for *in vivo* applications as therapeutic agents efficient even with a very low dose.

[Poster 26]

Identification and Validation of novel targets associated with metastasis using Chromophore-Assisted Laser Inactivation (CALI)

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Xerion employs its functional proteomics technology for the systematic functional analysis and validation of proteins in cell-based assays. The centerpiece of the technology platform is based on Chromophore Assisted Laser Inactivation (CALI), which provides a temporal and locally restricted protein knockout. CALI has been proven to specifically destroy protein function using dye-coupled specific antibodies and low energy laser irradiation. The mechanism involves the targeted induction of photochemical modifications at functional sites of the protein.

Xerion's technology reveals its full potential in a "disease-driven approach" which allows the simultaneous identification and functional validation of unknown targets. In this process, combinatorial antibody libraries are used to raise antibodies against complex protein mixtures, which are derived from diseased cells or tissues. This is followed by screening these antibodies with CALI in a disease-relevant functional assay. Antibodies that produce functional hits are subsequently used to immunoprecipitate target proteins, which are then identified by mass spectrometry.

Here we present the results of the application of this disease-driven approach to identify and validate targets involved in selected steps of the metastatic process like adhesion and invasion. This includes the isolation of single chain (sc)Fv-antibody fragments selected against a human fibrosarcoma cell line via phage display. These scFv were then tested for their ability to mediate inactivation of cell adhesion and invasion monitored by appropriate assays. Single chains Fv leading to "hits" were subsequently used to identify the respective target protein. Based on this integrated approach several new metastasis-relevant targets have been identified.

[Poster 27]

Preparation of the mouse and human chimeric antibodies to hypervariable region 1 (HVR1) of Hepatitis C virus

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Two broad cross-reactivity, high specificity and high affinity monoclonal antibodies (2P24 and 15H4) to HVR1 of HCV were obtained and characterised in our previous study (Li et al 2001. J. Virol. 75:12412). These antibodies captured 81% of HCV strains from unselected patientfls plasmas and blocked HCV binding to Molt-4 cells in a dose-dependent fashion. In an attempt to use these antibodies for passive immunisation of HCV infected patients or as prevention of HCV infection, these antibodies were partially humanised. The variable regions of MAbs 2P24 and 15H4 were individually grafted to a human IgG1 kappa constant

region. Genetically modified chimeric antibodies conserved a binding ability similar to that observed in the original murine MAbs. The chimeric antibodies produced in GMP standard will be further characterised *in vitro*, functionally tested in HCV-infected animals, and evaluated for clinical phase I in volunteers.

[Poster 28]
Tumour detection from hybridoma produced c-myc antibody
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The use of hybridoma, with production of monoclonal antibody is of profound significance in relation to their role in tumour detection. Study of retroviruses provided the first evidence of the involvement of specific genes in the process of tumourigenesis. Additional gene sequences, viral oncogenes (v-oncs) in their genomes are shown to be responsible for both tumour induction in animals, and trasformation of cells in culture. There are about twenty such transforming v-oncs, reported till date, which have been isolated from different retroviruses and are capable of inducing neoplasms in animals. These cellular transforming genes, referred to as cellular Oncogenes (c-oncs) arise within the cell as altered forms of normal cellular genes, the proto-oncogenes (P-oncs).

One such gene, belonging to the nuclear group of oncogenes, c-myc, has been implicated in the control of growth and differentiation of the cell. C-myc is a viral oncogene of avian myelocytoma virus mc-29 origin, related to a cellular homologue. The expression of c-myc gene and its protein product, a 62,000-dalton phosphoprotein is a subject of intense investigation.

In the present study, c-myc oncogene product has been studied in several human tumours using its antibody produsedby hybridoma as a molecular probe with immuno-histochemical methods. Almost all tumour tissues stained showed intense to weak nuclear staining of malignant cells.

[Poster 29]
High efficiency creation of human monoclonal antibody-producing hybridomas
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The native human antibody repertoire holds unexplored potential for the development of novel monoclonal antibody therapeutics. Techniques that fuse immortal cells and primary B-lymphocytes have been inadequate for the routine production of hybridomas that secrete human monoclonal antibodies. We have found that ectopic expression of interleukin-6 in the SP2/0 murine myeloma cell line improves the ability of this cell line to form stable heterohybridomas with primary human B-lymphocytes. The hybrid cells form at a frequency comparable to that observed for the formation of murine/murine hybrid cells. They maintain secretion of human antibodies derived from the primary B-lymphocytes through multiple rounds of cloning. Using splenic B-lymphocytes from a patient immunized with a *Streptococcus pneumoniae* capsular polysaccharide vaccine, we have succeeded in creating hybridomas that secrete human monoclonal antibodies specific for *S. pneumoniae* antigens. These experiments establish that the SP2/mIL-6 cell line will enable the rapid generation of native human monoclonal antibodies.