Human Antibodies 16 (2007) 45–60
IOS Press

Poster Session

An optimised monoclonal anti-CD20 antibody with enhanced ADCC against tumor cells from B-CLL patients

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This study investigates the cytotoxic activity of the anti-CD20 monoclonal antibody (mAb) EMAB-6 toward cells from patients with B-Chronic Lymphocytic Leukemia (B-CLL). Treatment of B-CLL using a combination of chemotherapy and anti-CD20 immunotherapy (rituximab) leads to less efficient clinical response as compared to Non-Hodgkin Lymphomas. This has been related to the low CD20 levels expressed at the B-CLL cell surface. MAbs with an enhanced ability to lyse CLL cells could induce therefore better clinical responses. We have generated a chimeric anti-CD20 mAb, EMAB-6, which exhibits a low fucose content in the Fc region and has improved FcgammaRIIIA (CD16)-binding as well as FcgammaRIIIA-dependent effector functions. Anti-CD20 mAB, EMAB-6, induces a much higher (x 70) *in vitro* cytotoxicity against PBMC from B-CLL cells than rituximab.

In addition, it also elicits higher FcgammaRIIIA-mediated IL-2 production than rituximab when FcgammaRIIIA-transfected Jurkat cells were co-cultured with $CD20^+$ B-CLL cells. Improved FcgammaRIIIA-mediated functions was specially observed at low mAb concentrations in the presence of B-CLL cells but also lymphoma cells and Raji cells.This study strongly suggests that EMAB-6 is more efficient than rituximab on tumor cells that express low levels of CD20 and represents a promising drug candidate for the treatment of B-CLL.

Monoclonal antibodies in the diagnosis of *peste des petits ruminants* (**PPR**) **virus infection**

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Introduction: Peste des petits ruminants (PPR) is an economically important viral disease of sheep and goats, prevalent in several countries of Asia and Africa. The disease is commonly referred as "Goat Plague" due to severity of infections and the high mortality rate. India has about 190 million small ruminants. According to an estimate in India alone, PPR causes an economic loss worth US$ 39 million. Effective disease management of these animals is therefore essential through the use of efficient vaccine, disease diagnosis and treatment.

Morphology & Antigenic relationship of PPR virus: The causative agent 'PPR virus' belongs to the genus *Morbillivirus* of the family *Paramyxoviridae.* The genus *Morbillivirus*, includes important animal and human viral pathogens affecting large ruminants, small ruminants, canine and human population viz: PPR, Rinderpest, Canine Distemper and Measles virus. The genome of morbilliviruses is a single stranded-RNA, approximately 16 Kilo bases (Kb) long with negative polarity. It is divided into six transcriptional units encoding two non-structural proteins (V, C) and six structural proteins; the surface glycoproteins which include fusion (F) and haemagglutinin (H) proteins, the matrix

ISSN 1093-2607/07/$17.00

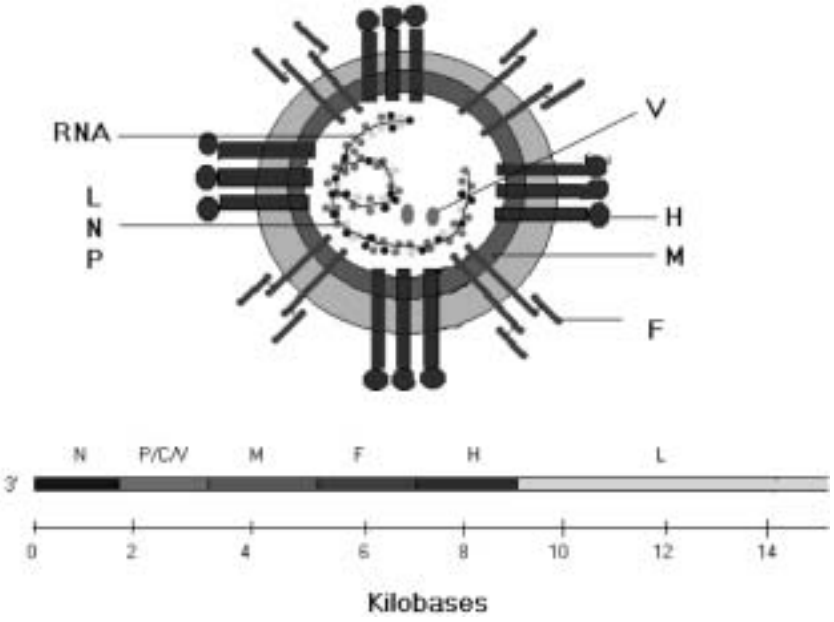

Fig. 1. Schematic diagram of a morbillivirus and it genome.

(M) protein, the nucleoprotein (N), the phosphoprotein (P) which forms the polymerase complex in association with the large (L) protein. Schematic diagram of a typical morbillivirus and its genome is presented (Fig. 1). Amongst these the haemaglutinin (H) protein located on the surface of virion is most dominant antibody inducers, while nucleocapsid (N) protein, an internal virion protein remains the most abundant antigen of virus origin. Therefore monoclonal antibodies to these proteins have been extensively employed in the diagnosis of PPR and other related diseases both for antigen and antibody detection.

Morbilliviruses share some common antigens with each other. The antibodies induced by any of these viruses partially cross neutralize or cross react each other. Monoclonal antibodies have also been extensively used for characterization of these organisms interchangeably for antigen profiling and establishing antigenic relationship.

Advantages of use of monoclonal antibodies in the diagnosis: Mouse monoclonal antibody based diagnostics have advantages over polyclonal antibodies to detect samples from domestic animals, wild animals, human and plant pathogens as none of these have close genetic relatedness with rodents. This is the reason that monoclonal antibody based assays are more specific than polyclonal antibody based test. Use of single enzyme conjugate (anti-mouse antibody conjugated with enzyme) is sufficient in competitive-ELISA as well as in sandwich-ELISA for antibody and antigen detection in clinical samples originating from different species of animals/human being. On the other hand polyclonal antibody based Indirect-ELISA requires use of enzyme conjugates originating from various species. Unlike human being monoclonal antibodies are not commonly used as therapeutic antibodies in case of livestock especially in developing world due to low cost of animals.

Antinucleocapsid (*N*) *monoclonal antibodies and their diagnostic application: Application in research.* Monoclonal antibodies to anti-nucleocapsid protein have been most widely used to characterize the morbilliviruses. 'N' gene is located towards 3' end in the viral genome; therefore it is the most abundant antigen in viral inoculums prepared. It seems during screening of hybridoma the MAbs to other proteins are out numbered and majority of the clones we get is directed against nucleocapsid [2]. The antigen to this protein is so much abundant in clinical materials as well as cell culture derived antigen that some times the sensitive antigen-antibody reaction has given false positive indication due to prozone phenomenon at the peak of infection/antigen excretion . This has been observed specially with the use of biotinilated monoclonal antibodies as detection antibody, which shows high degree of sensitivity in terms of quantity of antigen detection [4,8].

Application in diagnosis/antigen detection. The monoclonal antibody based sandwich-ELISA devel-

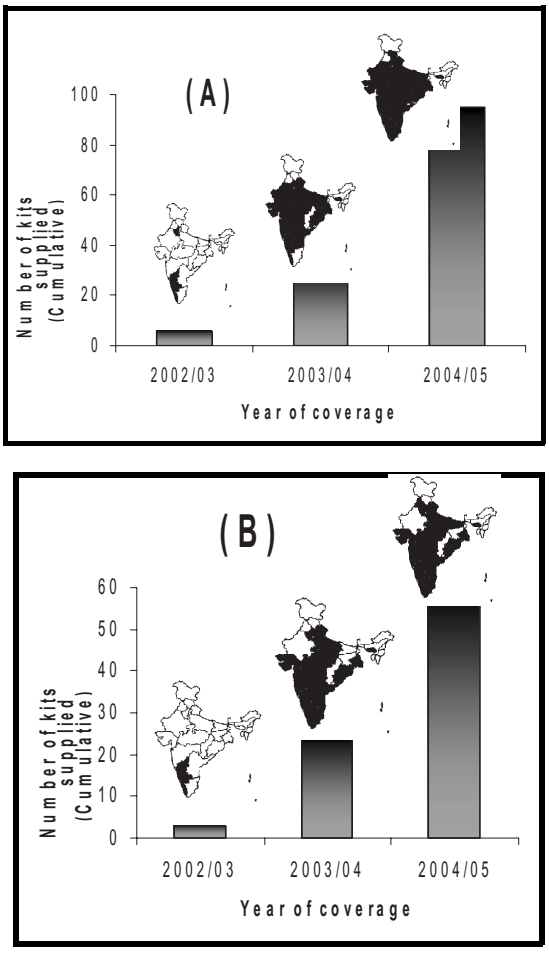

Fig. 2. Growing impact of PPR competitive-ELISA (2A) and Sandwich-ELISA (2B) kit developed by us in India. By now the diagnostic kits are being used in almost all parts of India.

oped by us using anti "N" MAb as detection antibody is an extremely simple and convenient system for routine diagnosis of PPR [4]. It is characterized by low cost of production ($1/50^{th}$ of the other diagnostic kits) and devoid of prozone phenomenon. Using a PPR virus specific monoclonal antibody directed against most abundant antigen we have also developed a cell-ELISA for the infectivity titration of PPR virus in cell culture with an extremely good correlation with cytopathic effect (CPE). Such assays will be especially useful for infectivity titration of viruses which are especially non-cytopathogenic in nature like swine fever virus and rabies virus in cell culture.

Anti-haemaglutinin (*H*) *monoclonal antibodies and their diagnostic application:* Use of monoclonal antibodies directed against haemaglutinin protein has served as a boon for the assessment of antibodies (sero-surveillance and sero-monitoring) against rinderpest and PPR viruses using single assay as competitive-ELISA [1,3,5,7]. These tests are based on the principle that binding of monoclonal antibody to antigen is inhibited in the presence of specific antibody in test sera samples. The antibodies from test sera occupy the epitopes present in the antigen, thereby not allowing monoclonal antibodies to bind to antigen. This results in reduction of the colour development, which indicates positivity of sera samples. Competitive-ELISA tests based on monoclonal antibodies to "H' protein have become more popular as they are based on virus neutralizing monoclonal antibodies against immunodominant epitope, which correlate well with virus neutralization assay the gold standard test. Further "H" protein is the Ist protein to be encountered during host pathogen interaction. Unlike HIV & Foot & Mouth Disease virus fortunately morbilliviruses of ruminants do not change frequently at genomic and antigenic level. This quality of virus allows us to use same vaccine and diagnostics in wider geographic areas of the world and also over a longer period of time.

Diagnostic kits developed using PPR monoclonal antibodies: Two diagnostic kits namely sandwich-ELISA kit for clinical surveillance and competitive-ELISA kit for serological surveillance have been developed using one each of anti "N" and anti "H" monoclonal antibodies [4,5]. The monoclonal antibody based diagnostic kits developed by us have been extensively used as gold standard tests for developing highly sensitive assays like PCR-ELISA , RT-PCR, virus neutralization test, competitive-ELISA from other source, Indirect-ELISA and competitive-ELISA using alternate monoclonal antibodies. Such an extensive comparison is rarely found with the diagnostic kits in the market. They have been validated from all the angles. The kits have become an indispensable tool for PPR research in India with growing popularity in India (Fig. 2A,2B) and the neighboring countries like china, Pakistan, Nepal etc. The diagnostic kits in addition to specialized diagnosis will be very useful in the demonstration of ELISA techniques, Radio-immunoprecipitation assay, virus neutralization test for teaching and training to the students.

Commercial aspects of monoclonal antibody based Diagnostic kits: Commercial production of diagnostics in developing countries do not have very good fate as on today. The manufacturers mainly are interested in vaccine manufacturing as it provides more scope for business. Further production of advanced diagnostics is relatively a more technical job than vaccine. Therefore with a manpower constraint, less initiative is tak-

en. This has resulted in the monopoly of the diagnostics by some manufacturers of diagnostics worldwide. It is therefore suggested that some international players must come forward to takeover the manufacturing of PPR diagnostics. Global Rinderpest Eradication Programme (GREP) is going to be over. India has already started FMD control programme. The next disease is likely to be PPR for control and eradication programmes. We have authentic baseline data [6], diagnostics and vaccine for PPR control programme.

The possibilities for transfer of technology/ commercial manufacturing of monoclonal antibody based diagnostics can be either marketing alone or manufacturing as well as marketing. In the former situation the production of critical reagents will be carried out at the parent laboratory. Commercialization of PPR diagnostics at international level will avoid monopoly of the existing manufacturers, thereby leading to benefit to end users in this era of globalization. Standard operating procedures (SOPs) have been developed critically for the production and evaluation of diagnostic reagents for day to day works.

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A photo-micrographic account of the sequential morphogenesis of hybridoma development

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ABSTRACT: Monoclonal antibody [1,2] development has become these days the bedrock of good medical and scientific practice. Most of the diagnostic and therapeutic works encourage good quality monoclonal antibody production. It is therefore necessary to pay much more attention to the health of these antibody-producing cells (Hybridomas). Good supervision and therefore careful monitor of these cells from the stage of fusion till confirmation that they are churning out indeed robust antibodies is absolutely crucial.

What is being done in this paper is to set out, as much as possible, the steps which one needs to pay attention to, develop a cute sense of healthy cell recognition until the stage that these (cells) are ripe enough for freezing down -in such a way that they can be easily re-suscitated, with high level of confidence of re-establishing production of quality monoclonal antibody. George Köhler and César Milstein [3] described the first derivation of monoclonal antibodies (mAbs) of defined specificity in 1975 and, for their work, were awarded the Nobel Prize in Physiology and Medicine in 1984. A variety of methods have been used to fuse, grow, select, and clone hybridomas since the original publication. What is considered most important is the plate and medium in which these cells grow and harvested. Methyl cellulose & gelatine -coated plates are among the few noted. We simplistically used IWAKI products and medium manipulation to produce quality hybridomas. This description is the first of its kind identified in published works.

Methods: The crucial steps in the development of Hybridoma are as outlined graphically in Fig. 1. This involved the selection of female mice, immunisation of mice (in this case the choice of adjuvant is important. TiterMaxGold [6] was employed instead of the classical Complete Freund's Adjuvant). The latter has potential problems, some of which are: a.) incitement of ulcerative granulomatous lesions at injected sites and therefore a cause of spurious antibodies which can interfere with the interpretation of the harvested monoclonal antibodies [7,8]. b.) repeated injections to enable

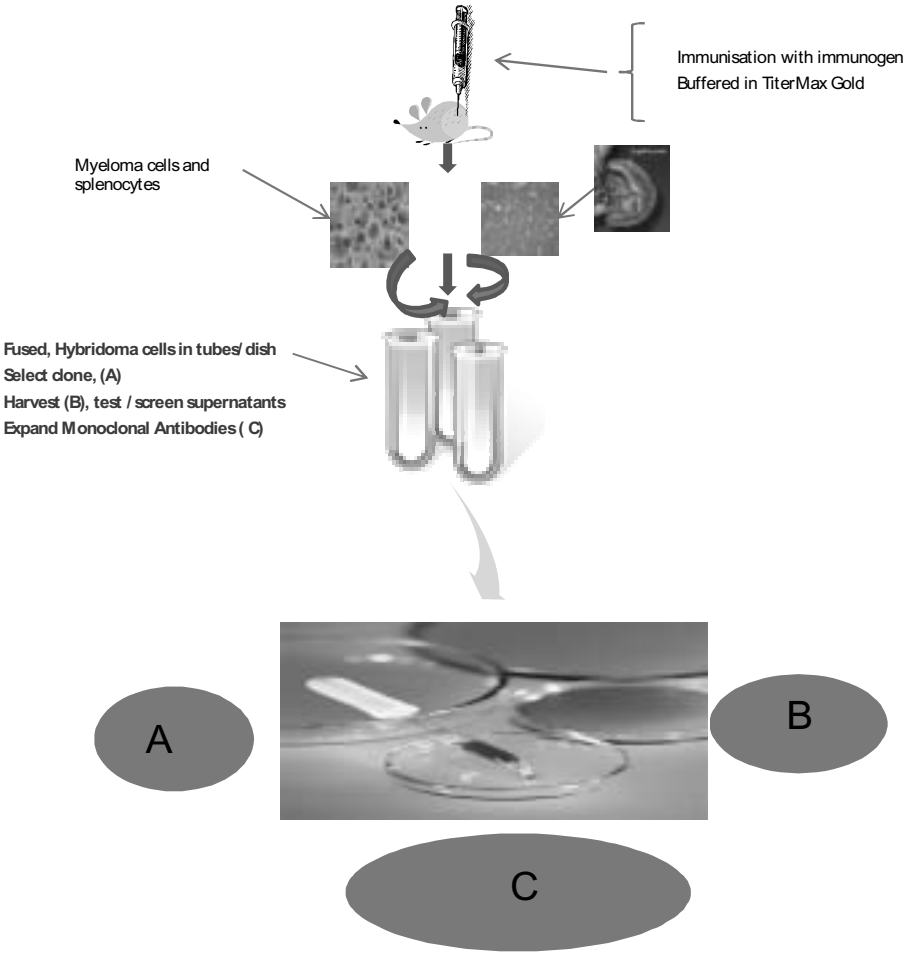

Fig. 1. MAb production commences with immunisation of mouse (4-6 weeks). Fusion with resultant production of Hybridomas 28days, at least. Selection, cloning and subcloning to expansion of choice mAbs 56 to 180days at least.

achievement of optimum antibody (polyclonal) stimulation in the mice. TiterMax Gold invariably requires one (1) injection only. This is adequate enough to provide more than enough antibody level at pre-fusion stage of splenocytes and choice Myeloma cell lines. In our case a booster dose was given and two (2) weeks allowed prior to sacrificing mice.

Thus the steps employed are as follows:

a) Immunization of mice,

b) Confirmation of optimal stimulation of antibody production in mice from tail & heart bleed (measured by ELISA, Chemiluminescence and W. Blotting),

c) Sacrifice of mice and removal of spleen, fusion of splenocytes to appropriately 'primed 'mouse Myeloma cell lines (NSO & Sp2, both treated with 8-Azaguanine) in a 96 well plate. Both Myeloma cell lines were employed in separate fusion to enable comparison of eventual yield of developed hybridomas. We did not confirm any advantage of NSO cell lines over Sp2, although the former did show steady robust growth [9,10].

Fusogenic agent of choice is Polyethylene Glycol (PEG) [5]. All these are carried out under strict aseptic condition in microbiological safety cabinets, class 1/Ii.

Results and discussion: The fused/developed cells (hybridoma) were treated as per standard protocol s [11,12,13]: Hybridomas were 'fed' in HAT (Hypoxanthine Aminopterin Thymidine) medium for the first fourteen days (D1-14); then in HT medium for the subsequent fourteen days (D15-28). The 'developed' hybridomas were then allowed to grow in 'standard' prepared medium of fetal calf serum, RPMI/DMEM or Glutamax (www.invitrogen.com). Appropriate supplements of Glutamine and prophylactic antibiotic of penicillin and streptomycin added as required.

Fig. 2. Supernatants from hybridomas showing robust Consistent growth were used as sources of Hybridokines (cytokines/chemokines) secreted From these cells invariably affect, positively the Growth of cells and quality of produced mono-Clonal antibodies.

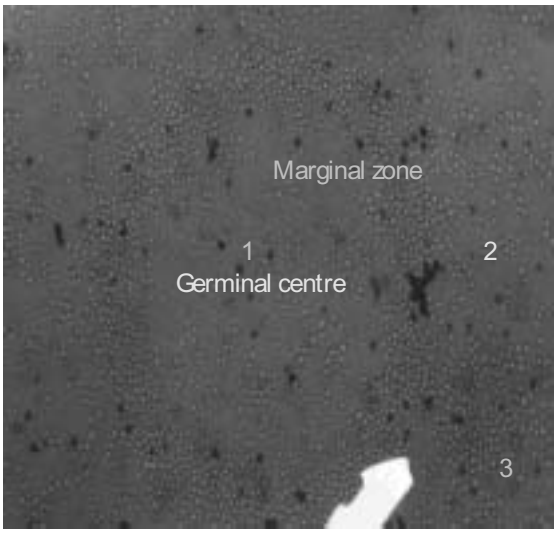

Fig. 3. Whoris of Hybridoma cells (at least 3) of such circularly arranged cells can be noted. Possible 'germinal centre' with characteristic paucity of cells and profusion of cells at the marhinal/submarginal zone areas.

Photomicrographs were sequentially taken from Day 1 through to Days28, and to Days 56.

A Steady evolution of fused golden, glistening cells noted from a 'cloud' of dark cells. The fused cells are characteristically polymorphic in nature. The sizes and shapes ranged from small cells, no different from typical lymphocyte to large round or oval shaped cells. Cardinally, all these cells have smooth cytoplasmic outline. No evidence of 'ragged' cell membranes noted. Most of these cells evolved as clusters and eventually arrayed in a lymphoid follicle fashion (Fig. 3). This is interesting because it does suggest that hybridomas are indeed lympho-plasmacytoid in nature and would perhaps have a lymphoid follicle with a mantle area and germinal centre.

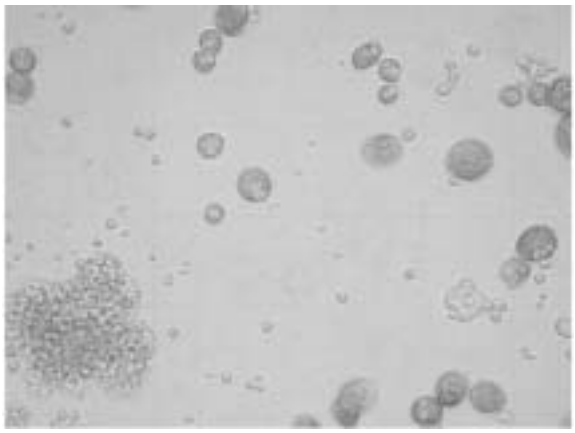

Fig. 4. Eovling Hybridoma cell lines. Note the degree of Polymorphism and 'clustering' tendencies.

Some of the cloud of dark cells persisted and eventually over the eight week period seem to disappear. This might be the unfused splenocytes and Myeloma cells.

After D28 some of these hybridomas were frozen, re-thawed and grown on a feeder raft of mouse fibroblasts. The pattern of growth as noted post-fusion was repeated, with golden cells evolving from the collection of dark cells. Whorls of cells circumferentially arrayed in a pattern no dissimilar to a lymphoid follicle with a germinal centre (filled with few large cells) and a mantle zone. Streaks of fibroblasts noted. The fibroblasts indeed provide good raft of feeder cells with a rather better profusion of growth. Better growth also noted with cells grown in serum free medium.

CONCLUSION: This is so far the first photomicrographic account on Hybridoma development. The most important stage in this process is the immunization program [4], using appropriate adjuvant (in this case TiterMax Gold) to ensure excellent antibody generating response in the mouse.

The medium, flasks and plates utilised are also critical. Lastly the regular/'religious' surveillance of the health of hybridomas is equally important. These are very 'fragile' cells and need utmost care for them to 'work 'for you.

USEFUL SITES AS PROVIDED BELOW

www.stemcell.com/technical/28411_clonacell-HY.

D1-7 post fusion. Confirmation of dark cloud of fused cells. Note the early glistening of hybridoma patch at 7 o clock.

Hybridoma cells showing profusion of growth on mouse fibroblasts as feeder cells.

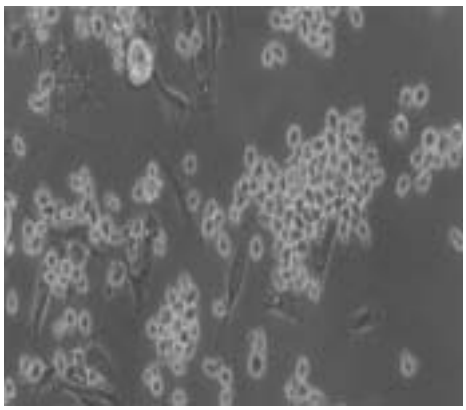

Fig. 5.

Promoters of Good Hybridoma growth:

Optimizing Immunization program
Nature of Medium: Serum Free Medium (SFM) tends to support growth
of hybridomas better than Serum-rich Medium (SRM)
Good adherence to strict fusion protocol, especially with promotion of
Feeder cells/layers- Thymocyte or mouse fibroblasts
Hybridokines. This includes the supernatant from 'ripe' Hybridomas
Flask/Well plate configuration.-special coatings (in this particular project IWAKI flasks were used throughout. For advantages visit: www.isisco.ie/isis/Files/Iwaki.pdf
Freezing methodology-quality of hoods used.
Antibiotic prophylaxis
Daily surveillance
Always look out for infected cells and cells stalling in growth.

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Chemiluminescence is a choice method in the preliminary stages of screening tail/heart bleeds and hybridomas in the development of monoclonal antibodies

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Table 1
W. blotting results of Tail bleed. Comfirm a clear optimal respose, post immunisation With HL Antigen, used as immunogen. This was obtained following the first dose with Immunogen, buffered in Titermax gold

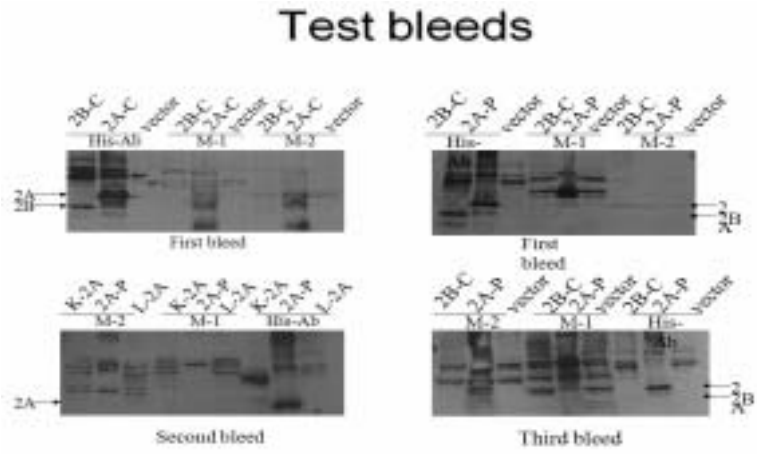
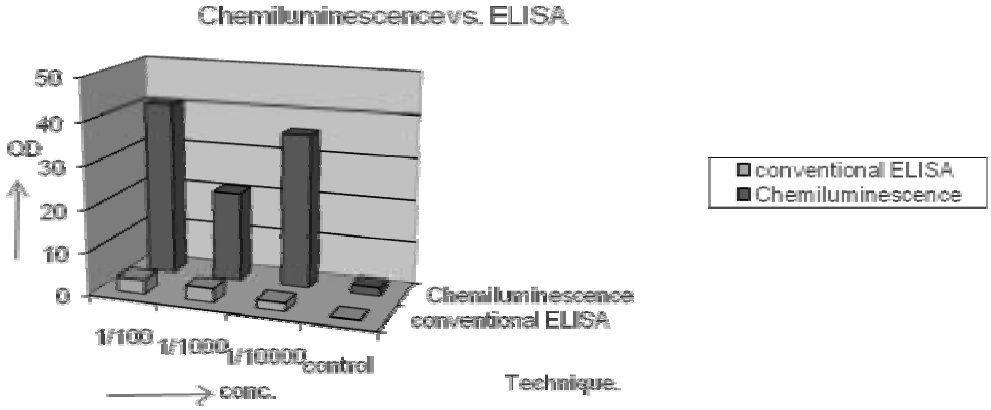


Table 2
Below confirm the superiority fo chemiluminescence over ELISA. This also affords a large batch screen of tail bleeds and very reliable, reproducible results



Chemiluminescence is more informative in rapid screening, and picking up low antibody concentrations that would have been 'missed' or difficult to interpret with 'ordinary' ELISA. Serial measurements for qualitative work would employ usefulness of this technique.

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Abstract: Immunological methods [1,2] have played a pivotal role in the screening of hybridomas as a stepwise approach in development of monoclonal antibodies (MAbs). Conventional ELISA methodologies have traditionally been used and invariably employed at every stage in the process of generating specific MAbs. As part of a strategy in developing novel antibodies to the human endogenous retrovirus, HERV-K10, and to Hodgkin's Lymphoma (both EBV and Non-EBV infected) a number of techniques were explored including chemiluniminescence, Western blotting and immunocytochemistry. The results obtained confirmed that chemiluminescence [7] provided an effective tool in precisely selecting-out, i) highly reactive hybridomas, ii) widening the scope of hybridoma selection at screening/characterisation stages (that encompass post-fusion and subcloning stages) and iii) At pre-fusion stages: tail bleed and heart bleed evaluation for adequate responses to immunogen. Chemiluminescence also proved cost-effective whilst Western blotting, although accurate, was most laborious and expensive.

Methods: The techniques employed and the comparative analyses had been discussed under the outlined headings:

Tail and Heart bleed testing for antibody production, post-immunisation: Development of monoclonal antibodies against Hodgkin's lymphoma by selecting

Table 3 and 4
Comparison of various immunological techniques

CHEMILUMINESCENCE
Shows higher , clear reading, and the difference between poor response is easily discerned.

20/06/03 chemi

Tail Bleed	Lysate KMH2	Lysate Herv. K10env	M678 A8	M670 A7
1/100	14.37	0.777	2.04	3.02
1/1000	13.545	0.336	0.934	0.97
1/10000	0.649	0.310	0.297	0.3

Control: 2.0

ELISA (DIRECT)
A greater subjectivity at times in interpreting results which most often results in reactive mAbs. Being missed.

20/06/03 ELISA

Tail Bleed	Lysate KMH2	Lysate Herv-K10env	M678A8	M670A7
1/100	2.697	2.267	1.4	1.44
1/1000	2.227	2.01	1.0	1.807
1/10000	1.112	1.32	0.92	1.006

Control 0.232

out Hodgkin's – Reed-Steinberg's (HRS) Lymphoma cell lines, both EBV and non-EBV infected and against Herv-K10, gag and envelope (env) proteins [8,9].

Six female mice were chosen and immunised with lysates prepared of the above proteins as immunogens, buffered in TiterMax Gold. Tail bleeds were tested for optimal antibody productions; confirmed optimum after a single immunisation after four (4) weeks and boosted for 'hyper'-production of antibodies on 4th week. Animals sacrificed, on 6–8th week and splenic tissues teased; products (splenocytes/lymphoid cells) fused with Myeloma (plasma) cell lines (SP2 & NSo) [10]. Heart bleeds were tested for proof of maximum antibody production.

Chemiluminescence, ELISA and W.Blotting techniques chosen as immuno-assay techniques). Very evident that W.Blotting use should not be continued because of cost.

Hybridoma Screening, Isotyping, Monoclonal antibody purification, Validation and expansion of hybridomas: The other methods used have been discussed under Results, and include the following phases: Hybridoma Screening, Subcloning & expansion of clones, Isotyping, Monoclonal antibody purification and Validation.

Results and discussion: Tail & Heart bleed testing for antibody production, post-immunisation: The tables below explain the practical issues employing the listed immunological techniques of Chemiluminescence, ELISA and W. Blotting:

W.Blotting, though conclusively superior as a mode of screening at the tail and Heart bleed phase (prefusion) is still not economically a feasible option and by far the best method is Chemiluminescence, in terms of ease, affordability , economy and sensitivity of results.

Hybridoma screening: Although the above illustrates the superiority of Chemiluminescence over ELISA, Immuno-histochemistry is more revealing (Tables 3 and 4 and Fig. 1), but the labour and screening work make it practically impossible in standard laboratory work. This is also not feasible in routine industrial/research facility, Methods such as flowcytometry, might take the place of IHC; again expense is the key in deciding which method to choose in the preliminary stages of Hybridoma screening.

Subcloning and Isotyping phase: Monoclonal Abs. screened using Chemiluminescence and ELISA is more than two thousand (2000), which correspond somewhat to the number of HYBRIDOMAS obtained by fusion. Selections were made largely with Chemiluminescence and ELISA, as these were the most sound and convenient methods at the initial phases. A total of 54 Hybridomas obtained with Chemiluminescence; and greater than 100 with ELISA. After Subcloning and Isotyping (Isotyping done with ISO-KIT from Sigma and Hycult [5,7]). Sample of results as shown. Also the effectiveness of ELISA[1] as shown below.

Many of the very reactive clones came from the designate Clone 'M', confirming effectiveness of Chemiluminescence (see Table 2).

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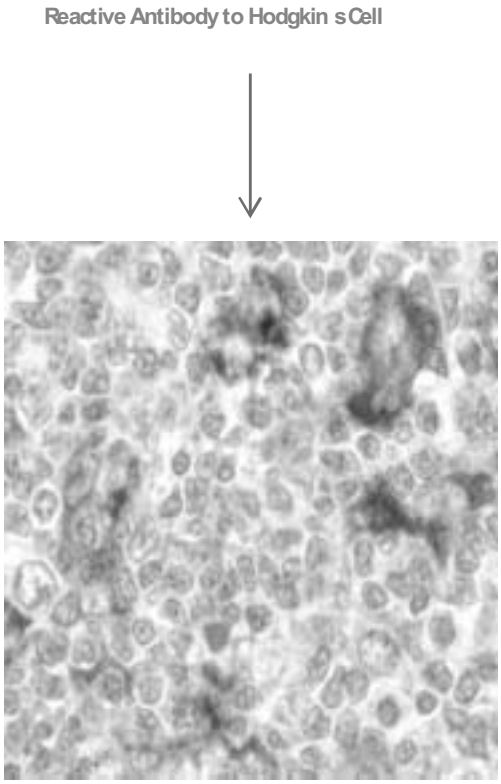

Fig. 1. Shows clear reactive, mAbs and therefore ideal for rapid screening; but rather impractical and too labour intensive.

Cost Implications: Cost implications limit the use of more 'fine' immunological tools in initial screening work. As shown below a screen using w.blotting for limited no. of samples are about 10 times the cost of ELISA technique and 8 times the cost of Chemiluminescence (*Table provided in Poster session*). The cost implications in developing monoclonal antibody is beyond the scope of this study, however the best way to evaluate the cost is by taking into account the quality of the service producing monoclonal antibody for research, clinical and diagnostic work. This therefore will encompass the need for more effective immunological techniques including Immunofluorescence, flow cytometry, more frequent use of w.blotting techniques, and undoubtedly chemiluminescence. The cost of the aseptic unit becomes even most paramount in this regard, as this is the way of ensuring reproducibility and recoverability of hybridomas and maintenance quality production of monoclonal antibody.

Table 5
More mAbs were isotyped and screened by the ELISA method. Those obtained by chemiluminescence (designated as 'M') were some of the very reactive mAbs obtained From the program

Original clone	Designation after subcloning	Isotype
P4101	P4101	IgG1κ
P317	P317	IgG2bκ
P15a	P15a3	IgG1,2bk
P15	P152	IgG2bκ
P58b	P58b7	IgG3κ
	P58b81	IgG1κ
P1L	P1L12	IgG1κ
	P1L115	IgG1κ
M5	M5	IgG1κ
M5H	M5H	IgG1k
P514b	P514b17	IgG1,Mκ
	P514b27	IgG1κ
P54b	P54b2h	IgG2bκ
P53b	P53b	IgGκ, Mκ
M4	M4a	IgG2a, A,E,Mκ
	M4ei	IgG1κ
	M45	IgG1κ
	M49c	IgG1κ

Conclusions:

1. Chemiluminescence by far provided scope of choosing most reactive antibodies and widening the scope of selection of produced hybridomas, being a more sensitive technique than ELISA.
2. There is ease with ELISA techniques, but also great subjectivity especially with borderline results. There are also high levels of false positivities and higher baseline values (control) than CHEMILUMINESCENCE.
3. Immunoblotting techniques are most useful after a select monoclonal antibodies have been identified for future work, e.g. Investigating the expression in variety of test tissues
4. A combinational method of Isotyping (both ISOKIT-strip tests and Indirect/direct ELISA are confirmatory, especially as ELISA can be fundamentally a 'crude' technique.

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Comparison of seurm free medium and serum rich medium in hybridoma development at subcloning and isotyping phase and how it impacts on protein purification.also a choice method of reducing use of animals in scientific experiments

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Abstract: Serum-rich medium (SRM) [1] may have limitations in hybridoma production/monoclonal antibody (MAb) development. The search for alternatives have become necessary for many reasons: reduction in cost, limitation of exposure to unknown transmissible strains of virus in foetal calf serum (FCS) and reduction of non-specific proteins at protein purification stage. In this study, we have looked at the use of serum-free medium (SFM) [3,6] on monoclonal antibody development and the viability hybridomas after freezing and thawing. The observations suggest optimum ELISA/chemiluminescence data, suggesting an increased quality of hybridomas produced. Moreover, there was an increased growth of hybridoma cell lines, confirmed by viability and cell counting studies. Furthermore there was less labour at the protein purification stage. However, there was no guarantee of better protection against infection and in some cases there were difficulties with the maintenance of viability of frozen cells [4] following thawing and resuscitation. From the point of bio-safety, alternative media that is cost-effective should be explored.

Methods and results: Monoclonal antibody-producing (Anti-HERV-K10, Anit-KMH2/KMH2-EBV) hybridomas developed were subcloned and eventually characterised (Table 1). The subcloned hybridomas [6,7] were subsequently expanded and treated with various hybridokines, including supernatants from robustly growing hybridoma cell lines. At this stage of manoeuvring to optimise antibody production two groups of media were utilised; a Serum-Free Medium and Serum-Rich Medium. The hybridomas were initially grown in SRM and gradually weaned to SFM. A selection of flasks and plates were allocated for SFM and compared with the equal numbers of flasks and plates growing the same designated cell lines in SRM. The weaning was done commencing at 10% reduction of SRM and gradually tapering it to 100% over a period of 3 weeks. Cell were subsequently encouraged to grow in SFM for a further 3 weeks and eventually frozen down after the various experiments were carried out including a. Viability studies/Cell counting (using a new coulter counter equipment from Beckton-Dickenson aavailable In- house). b. ELISA /Chemiluminescence. c. Protein purification [8] with AKTA FPLC Chromatographic System (*from GE Healthcare and Biological HR Chromatographic systems*, *from Bio-Rad*), and d. Immunohistochemistry(IHC)

Table 2
Selection of subcloned hybridomas (and isotyped) used for the experiment

Original clone	Designation after subcloning	Isotype
P4101	P4101	IgG1κ
P317	P317	IgG2bκ
P15a	P15a3	IgG1,2bk
P15	P152	IgG2bκ
P58b	P58b7	IgG3κ
	P58b81	IgG1κ
P1L	P1L12	IgG1κ
	P1L115	IgG1κ
M5	M5	IgG1κ
M5H	M5H	IgG1k
P514b	P514b17	IgG1,Mκ
	P514b27	IgG1κ
P54b	P54b2h	IgG2bκ
P53b	P53b	IgGκ, Mκ
M4	M4a	IgG2a, A,E,Mκ
	M4ei	IgG1κ
	M45	IgG1κ
	M49c	IgG1κ

Media were obtained form the following company sites:

1. http://www.sigmaaldrich.com/Area_of_Interest/Europe_Home/UK.htm.
2. http://www.perbio.com/site/ProductLine/Products.asp?show=e&var=hyclone&poll=hyclone&stats=Serum-free%20media&cat=3&table=NewProduct.

Conclusion and discussion:

Table 1
Result following replacement of SFM with SRM. Evidence of better reactivities of hybridomas grown in SRM (See chemiluminescence and ELISA). No significant difference noted in IHC. HCL-Hybridoma Cell lines, SFM-Serum Free Medium-CDhybridoma(invitrogen), SRM-Serum-Rich Medium-F2442, RPMI-Sigma, RPMI(Glutamax)-Invitrogen

Tests / Media	SRM	SFM without feeder cells	SFM plus feeder cells	SRM plus feeder cells
HCL in 25ml flask	MBL4, P15, P15a. P4101, 514b, 53b, p58b. L10	MBL4, P15, P15a	NA	NA
HCL Sublconed Growth intensity(+ or -)	NA	Poor yield	MBL4, P15,P15a	MBL4, P15, P15a,p4101, 514b,53b P58b, L10,
ELISA (OD)	NA	NA	>2.0	>1.5
Chemiluminescence			>300	>220
IHC- (with supernatant)			75% vv of supernatant ++	100% of supernatant ++
Purified Pro-			same	same

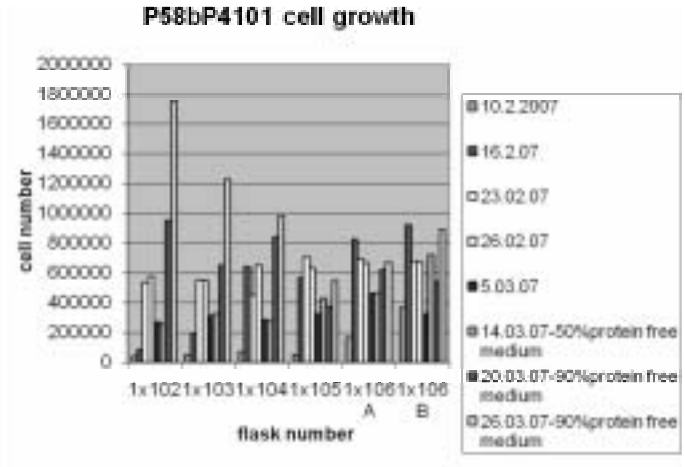

Fig. 1. Shows the variable growth of hybridoma cells at the concentrations in SFM indicated. The higher the SFM concentration, the higher the viability counts.

– We managed to expand and harvest good quality hybridomas in serum-Free medium. The cells grew better however in our hands when the SFM is supported by feeder layer of mouse fibroblast.

– Not all the Hybridomas we selected thrived in SFM. This is not unusual as some of these need a adjusting to the new medium(in this case mouse products) for effective growth.

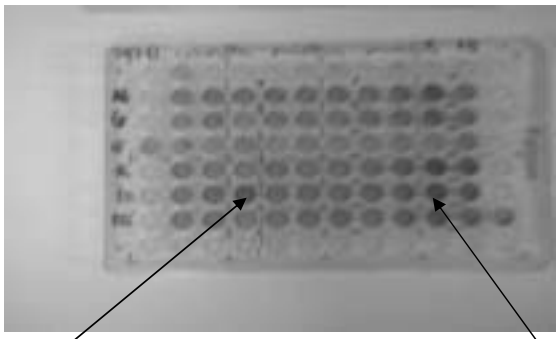

Fig. 2. The darker the wells the higher the reactivity and also reflects effective mAb production by hybridoma.

– The concentration of protein following purification did not differ significantly in both SFM & SRM.
– There are significant benefits using SFM on the whole, more pivotally providing a sure alternative to use of live animal in scientific experiment.Other benefits like: eliminating the need to pre-screen serum lots,simplified regulatory documentation,consistent media performance, reduced downstream purification challenges cannot be ignored.
– The odds are very much in favour of improving the use of SFM in future work. The cost was less with SFM than SRM. The provision was also more instant.

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An efficient in vitro antibody generation system using a hypermutating B cell line

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A chicken B cell line DT40 spontaneously mutates Ig genes during culture by activation-induced cytidine deaminase (AID)-dependent gene conversion and point mutation. Thus, cultured cell population of DT40 can be used as an antibody (Ab) library containing a wide variety of antigen (Ag)-specificity. To establish in vitro Ab screening system using DT40, we generated an engineered DT40 line whose AID expression can be reversibly switched on and off, thereby enabling ON/OFF control of the mutation machinery. In a DT40 line that was transfected with tamoxifen-regulated Cre recombinase, one AID allele was disrupted, and the other allele was replaced by the loxP-flanked AID construct. This engineered cell line named DT40-SW constitutes an Ab library when the cells are continuously cultured with AID on, while selected clones producing a desired Ab can be stabilized by switching off AID expression after tamoxifen-treatment.

By panning with Ag-conjugated magnetic microbeads, we isolated DT40 clones secreting Abs to vari-

ous Ags including 4-hydroxy-3-nitrophenylacetyl (NP) hapten, bovine serum albumin, human IgG and ssDNA. Interestingly, Abs to self-components like ovalbumin and hen egg lysozyme were also obtained in this system, thus suggesting that the DT40-SW cell population retains Ab repertoire that may be eliminated in vivo by immunologic tolerance.

Primarily isolated NP-specific clones were further cultured for diversification for several weeks followed by secondary selection. Repeated cycles of these treatment led to the generation of clones producing Abs with higher affinity to NP. Thus, Ab affinity can be improved in the DT40-SW system by affinity maturation mechanism that is basically similar to that occurring *in vivo*. In conclusion, DT40-SW is useful in establishing an efficient *in vitro* Ab generation system.

Are Protein A-Sepharose and Prosep-vA Ultra suitable and stable matrices for plantibody HB-01 purification from tobacco extracts?

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The purification of antibodies expressed in plants is an easy theoretical task but difficult to achieve due to the complexity of leaf extracts and the low expression level of a great number of plantibodies. In general, plantibody purification can be performed using the same purification matrices reported for antibodies purification from cell cultured supernatants and ascitic fluid, such as Protein A-Sepharose. Nevertheless several authors reported the blocking of the affinity columns very often, mostly if the extraction and clarification steps are not robust enough and low adsorption capacity, recovery and flow rates. To overcome these operational problems several matrices based on the porous glass technology have been more recently produced. In this work, we evaluated the suitability and stability of the Protein A-Sepharose fast flow and Prosep-vA Ultra for the plantibody HB-01 purification from tobacco extracts in 50 cycles. Purified plantibody HB-01 preparations showed purity over 90 and 95% by HPLC-GF and SDS-PAGE respectively with independence of the affinity chromatography matrix used. The recovery of Prosep-vA Ultra in 50 cycles was $66.14 \pm 24.0\%$, meanwhile Protein A-Sepharose fast flow recovery in the same number of cycles was $84.79 \pm 15.10\%$ but a different processing strategy had to be applied because of the fast column blocking. As conclusion of these experiments it can be assured that Protein A-Sepharose fast flow and Prosep-vA.

Comparison of different ligand densities in immunoaffinity chromatography of the plantibody HB-01 coupled to Sepharose CL-4B to purify the rHBsAg

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This paper evaluates the immunopurification behavior of a plantibody HBsAg specific plantibody coupled to Sepharose CL-4B at different ligand densities. Results shown no significant differences in the adsorption and elution capacities, and rHBsAg recovery of immunosorbents at 3.43, 4.45, and 5.31 mg/mL of ligand densities compared to its mouse-derived mAb counterpart consistently used in the rHBsAg purification process. Therefore, plantibody ligand densities higher than 3.43 mg/mL do not improve the immunopurification behaviour of this immunosorbent, increase the antibody consumption and the Hepatitis B vaccine cost. Immunosorbent of 2.23 mg/mL of ligand density demonstrated a poor performance. The IgG leached detectable level never exceeded the approved limit (3 ng IgG/μg rHBsAg). Values close to this limit were only observed at the ligand density of 5.31 mg/mL and 2.27 mg/mL. In the case of the ligand density of 2.23 mg/mL the IgG leached value was high (2.90 ng IgG/μg rHBsAg) due to the low level of eluted antigen. In conclusion, it supports feasibility of using this plantibody at 3.43 mg/mL of ligand density for large-scale immunopurification of rHBsAg for human use avoiding the biosafety and ethic concerns the massive use of animals for this purpose.

Evaluation of a Plantibody HB-01 purification strategy at different scales

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Transgenic plant investigations focus on expression fine-regulation and protein large-scale purifica-

tion. In this article, preliminary experiments were done at analytical-scale (<10 kg of biomass) to decide best plantibody HB-01 purification strategy. Once it was assumed, purification efficiency was then analyzed at different scales (10-600 kg of biomass). Plantibody SDS-PAGE and HPLC-GF purity were always over 90%, yielding 9.9±6.2-18.6±0.9 mg IgG/kg of biomass and from 39.9±7.9% to 48.7±2.1% of recovery. Significant differences were not observed in the evaluation of these parameters. Plant DNA contents was <3.18 ng/mg IgG, which is considered very low for the plantibody HB-01 application in immunochromatography.

Evaluation of a *Nicotiana tabaccum L.*, variety in a green-containment and natural zeolite for safe and consistent biomass and leaf soluble protein production for human use

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The *Nicotiana tabaccum L.*, variety Habana-92 productive potential using a green-containment and natural granulated zeolite was studied to contribute valuable information about plant selection for transgenic protein production. Seedlings were grown in 1320 meter^2, and leaves were harvested 6-7 weeks after transplanting to be weighed and macerated. The comparison of morpho-agronomic characters of this variety cultivated in zeolite confirmed similarity in qualitative characteristics but differences in plant height and leaf weight with those reported in natural soil and open field. First-round results using a random block design demonstrated that 15 plants/m^2 was the optimal plant density to perform pilot-scale experiments. The highest biomass and leaf total soluble protein (LTSP) production was observed during the spring season, thus demonstrating significant differences between the seasons of the year (*P-values*<0.05). Biomass yield showed a moderately strong correlation with the greenhouse temperature ($r = 0.91847$) and humidity ($r = 0.89182$), whereas the correlation coefficient for LTSP production also shows a moderately strong correlation with temperature ($r = 0.83228$) but a weak correlation with humidity ($r = 0.58570$). Summarizing, this tobacco variety allows the production of 100.77 tons of biomass and 3.91 tons of LTSP per hectare/year with independence of natural soil, regions and climate conditions. The evaluation of two hypothetic transgenic protein expression level sceneries would permit the production of 3.91 (expression level, 0.1%) or 39.15 kg/ha/year (expression level, 1%) in leaf extract. In conclusion, these results corroborate that this tobacco variety could be used for consistent and safe production of transgenic proteins in a green-containment and natural zeolite.

The establishment of a documentation system and quality controls for the plantibody HB-01 production used in the rHBsAg purification for human use

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The plantibody HB-01 is a plant-derived antibody expressed in tobacco plants to overcome the constrains of the massive use of animals for the production of the monoclonal antibody (Mab) CB.Hep-1, routinely used for the immunopurification of the recombinant Hepatitis B surface antigen (rHBsAg) for the Hepatitis B vaccine production. The subject of this work was the establishment of a documentation system to assure the required information of all activities related to the manufacture procedure, quality specifications of raw materials, solutions, components, intermediate and finished products and the standard operation procedures (SOP). Authors also described the quality control strategy to demonstrate the quality of the rHBsAg purified by means of Mab CB.Hep-1 and plantibody HB-01 immunosorbents. This documentation system, structured in four batch master files, 98 quality specifications, 67 SOPs, 5 inspection points, 16 process and quality controls allowed the demonstration of the biocomparability between rHBsAg purified by both immunoaffinity matrixes. In conclusion, this paper is the first report where a complete strategy of documentation and control was established for the production of a plantibody used as immunoreagent in a human use drug substance production.

Green protein engineering and transgenic plants: is there a link between them?

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"Green Engineering" is an emerging area of chemistry. It can be defined as a part of chemistry that applies process engineering tools to design sustainable and safe chemical processes. It involves the integration of new environmentally friendly chemical routes and technical innovation in order to achieve green process development. On the other hand plant bioengineering for biopharmaceutical production for human use has expanded in recent years because of advances in novel production systems and the need for very large quantities of therapeutic proteins. However the public acceptance of this kind of product is very poor due to unawareness and the non–implementation, in some cases, of cleaner productions that takes advantage of wastes for avoiding their release without a proper processing and inactivation. The relationship between these areas will help to achieve a public acceptance not seen in transgenic products before and it will contribute to products recycle in a sustainable development platform. In this work are exposed the results of a possible relationship with the potential use of plantibody HB-01 extraction wastes. The plantibody HB-01 was purified from tobacco plants with a yield of 25 mg/biomass kg, a 60% of recovery and 90% of purity by SDS-PAGE and HPLC-GF. In parallel it was evaluated production of multiparticule boards from tobacco wastes for furniture manufacture and interior decoration at a lower cost than traditional play-wood production technology. Due to the generation of large quantities of stems and leave wastes during tobacco processing for plantibody HB-01, the combination of both processes to accomplish a cleaner and efficient production, was proposed. As well, the utilization of other wastes was proposed in order to obtain nicotine, solanesol and antioxidants but further experiments are needed for these purposes. These results allow the prediction a green, promising and sustainable future for plant made pharmaceuticals.

Characterization of antibodies direct against the idiotypic VK chain of a HCV-related b-cell lymphoma

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Accumulating evidences support a role for hepatitis C virus (HCV) in the pathogenesis of human lymphoproliferative disorders: type II mixed cryoglobulinemia (MC), a chronic immune complex-mediated disease, and B-cell non Hodgkin's lymphoma (NHL). The idiotype of the B cell receptor (BCR) expressed on the B-cell clone surface

can be identified as a particular tumour-specific antigen. In previous studies we demonstrated that the BCR repertoire expressed by HCV-associated NHL is not random, with V1-69/V3-20; V3-7/V3-15, V4-59/V3-20 variable heavy (VH)/variable light (VL) chain gene combinations the most represented.

In this context the production of an anti-idiotype antibody, directed against a recurrent BCR rearrangement, could be useful not only in the patient who has supplied the receptor but also in other patients sharing the same rearrangement.

In this study we produce and characterized 5 monoclonal antibodies reactive against the VK (herein named VKgal) light chain expressed by the tumour sample of a patient with B-cell NHL, MC-II and HCV infection and that we have previously produced as a protein recombinant.

These antibodies are able to recognise Vkgal in Western Blot and showed a different affinity, towards the VKgal protein, in Elisa test. Furthermore, we used the epitope excision technique, combined with MALDI-TOF mass spectrometry, to identify the epitopes recognized by our monoclonal antibodies.

Our experiments have done indication regarding the immunogenic epitopes present in the VKgal chain. Vkgal structural 3D model identified the exposition of specific amino acid on the epitope region.

Data are useful to better understand the mechanisms of humoral immune response in the course of HCV-related B-cell proliferation, and to find common epitopes present on clonal B-cell of HCV-infected patients.