Functional recombinant antibodies against human chorionic gonadotropin expressed in plants

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Single-chain variable fragments, diabodies and chimeric antibodies (mouse variable domains and human immunoglobulin constant domains) were engineered by DNA recombinant technique and expressed transiently in tobacco leaves. The plants expressed the three types of antigen-binding moieties, accurately and faithfully. The yield obtained was 32 mg, 40 mg and 20 mg respectively, per kg of wet weight of leaves. The chimeric antibody had high affinity for human chorionic gonadotropin ($Ka = 1.9 \times 10^{10}\ M^{-1}$). All three forms of the recombinant antibodies expressed by plants inhibited the binding of hCG to receptor on Leydig cells.

SEROTHERAPY has been employed for life-threatening infections since the 1890s for tetanus, diphtheria and rabies. Initially antibodies raised in horses were employed, which limited the repeat use of such antibodies for therapeutic interventions owing to sensitization caused in recipients, to the heterospecies proteins. In recent years antibodies used as a last resort at the terminal stage of infections are derived from human sera prepared from hyper-immunized donors. These are consequently expensive. However for snakebites, horse continues to be the source of serum for therapy. The contention of this article is to propose and demonstrate that humanized therapeutic antibodies can be made by recombinant DNA route. Furthermore, plants can be used for expression of the recombinant antibodies. Plants offer several advantages. Besides being eco-friendly, plant-derived immunoglobulins would be expected to be devoid of harmful endotoxins and animal viruses present in the commonly used prokaryotic or animal cell culture expression systems. We describe the successful production of recombinant antibodies against the human chorionic gonadotropin (hCG), employing plants as an expression system. Also illustrated are the different constructs of antigen-binding fragments that can be engineered and their relative merits

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as functional antibodies. The data presented bring out the exquisite ability of plants to make antibodies. The plant machinery not only expresses the heavy and light chains of the immunoglobulin molecule, but also assembles them correctly to generate functional antibodies.

hCG is made by the human embryo before its nidation on the endometrium[1]. It has an essential role in the implantation of the embryo to initiate pregnancy. Antibodies against hCG prevent implantation in marmosets[2] and in humans[3], as women immunized with a vaccine generating antibodies against hCG do not become pregnant, have regular menstrual cycles and show no lengthening of the luteal phase[4,5]. Anti-hCG antibodies can thus be used for control of fertility. Ectopic production of hCG and/or its subunits has also been observed in several cancers. Malignant tumours of the bladder, pancreas, uterus and lung predominantly secrete the free beta subunit, especially at the aggressive stage of metastasis[6,7].

The use of antibodies, in particular monoclonal antibodies, in diagnostics is widely prevalent. Of late their use for therapy of cancer[8–11], autoimmune disorders[12], allergies[13] and prevention of transplant rejection[14], is gaining ground. Thus there is the need for large-scale production of antibodies at low cost. Bacterial expression systems are routinely used for large-scale expression of antibody fragments like single-chain variable fragments (scFv), Fab and diabody fragments, but these organisms are unable to produce multimeric full-size antibody molecules. Yeast[15] and insect cells[16] have been used, though to a limited extent, to express complex antibody molecules. Mammalian cell culture system for expression of antibodies is established, but is expensive in addition to the risk of carrying over contaminating infectious agents. We demonstrate in this communication the expression at high yields of functional anti-hCG recombinant antibodies in plants.

The starting point of this work was a mouse monoclonal antibody reactive with hCG with a high affinity ($Ka = 3 \times 10^{10}\,M^{-1}$) and specificity[17]. The genes coding for the antigen-binding regions, the variable domains, of the heavy and light chains of this antibody were isolated from the mRNA of the hybridoma cells which are primarily engaged in making uniquely this antibody. cDNA was prepared by RT–PCR, using primers specific for constant domain of mouse IgG1 and kappa chains. The immunoglobulin heavy and light chain variable domains were amplified by PCR and cloned for the construction of the following three types of antigen-binding molecules: (i) the single chain variable fragment[18] (scFv), where the variable heavy (V_H) and light (V_L) chain domains are linked together through an 18-amino acid flexible linker sequence and which can fold to form a single antigen binding site; (ii) the diabody[19], where the V_H and V_L domains are separated by a shorter 8-amino acid linker sequence, forcing the domains to pair with complementary domains of another such fragment, to form two antigen binding sites; (iii) the chimeric antibody, a full size antibody consisting of the murine V_H and V_L domains linked to the human IgG1 and kappa constant domains, respectively.

In order to determine that the constructs would be synthesized as recombinant antibodies (rAbs), scFv and diabody fragments were first cloned in a bacterial expression vector pSin1 (ref. 20), which has the His-6 tag and were expressed in *E. coli* (HB2151). For expression of the antibody fragments, the bacterial cultures were induced with 1 mM IPTG and 0.4 M sucrose, for 3 h at 28–30°C or overnight at the same temperature. The rAbs were obtained from the extract of bacterial periplasm (when induced for a period of 3 h) or were secreted into the culture medium on overnight induction.

The periplasmic extracts were prepared from cells harvested after 3 h of induction. The cell pellet was resuspended in 5 ml of cold Tris buffer (30 mM Tris, 20% (w/v) sucrose, pH 8.0) and EDTA added to a final concentration of 1 mM. The suspension was incubated at 4°C for 15 min with gentle agitation followed by centrifugation (8000 g/4°C/20 min). The supernatant thus obtained was removed and kept on ice. The remaining pellet was resuspended in 5 ml of 5 mM $MgSO_4$ and EDTA added to the suspension to a final concentration of 1 mM. This was followed by incubation at 4°C for 15 min and centrifugation as before. This supernatant was mixed with the first supernatant. The mixture of cold osmotic shock fluid was dialysed against PBS and affinity purified. The purification was carried out using Ni-NTA agarose (Qiagen) according to manufacturer's protocol.

The functionality of the rAbs was tested for hCG binding in direct-binding ELISA and in competition ELISA[21] against the parent mouse monoclonal antibody.

For the chimeric antibody, the heavy and light chain constructs were cloned directly in a plant expression vector, pSSH1 (ref. 22), since these would not be properly folded and assembled together in bacteria. In order to determine the comparative yields and functional qualities, the scFv and diabody genes were also cloned individually into the plant expression vectors. Figure 1 gives the constructs in the plant expression vector, pSSH1. These constructs were electroporated into *Agrobacterium*.

The *Agrobacterium* carrying the expression constructs was vacuum-infiltrated[23] into *Nicotiana tobacum* leaves. For the chimeric antibody, *Agrobacteria* carrying the chimeric heavy and light chain constructs were infiltrated either individually or together for expressing the full size chimeric antibody. Extracts from infiltrated leaves were prepared according to Vaquero *et al*.[24] and tested for hCG binding in ELISA, for analysis by Western blot or for affinity purification. The his-6-tagged plant-expressed scFv and diabody were purified using Ni-NTA matrix, as before. The full size chimeric antibody was purified using Protein-A matrix (Pharmacia) from leaves co-infil-

trated with the *Agrobacterium* carrying chimeric heavy and light chain constructs. The purity of the samples was analysed on SDS–PAGE and is depicted in Figure 2.

BCA reagent (Pierce) was used for quantification of protein concentration according to manufacturer's instructions, using bovine gamma globulin as a standard.

For capture ELISA, ELISA plate wells were coated with goat anti-human $F(ab)_2$ [GAH-$F(ab)_2$] or Fc [GAH-Fc] specific antibody (1 µg/ml). The leaf extract, diluted in phosphate buffered saline containing 0.05% Tween-20, was applied to the wells. Detection of the bound light and heavy chains was carried out by using GAH-$F(ab)_2$ alkaline phosphatase (AP) conjugated antibody and GAH-Fc-AP conjugated antibody, respectively. The substrate used for the AP label was 1 mg/ml *p*-nitrophenyl phosphate (Sigma) in substrate buffer. The absorbance was measured at 405 nm.

Bioneutralization capacity of the antibodies was determined by testing their ability to inhibit the binding of ^{125}I-labelled hCG to rat testicular receptors, following the procedure described by Pal *et al.*[25]. Bioneutralization capacity was plotted as percentage of inhibition of binding of hCG to receptor, against antibody concentration.

The plant leaves expressed all the antigen-binding molecules, namely the scFv, diabody and the chimeric antibody. Moreover, the yield of scFv was 32 mg of purified fragment per kg of fresh weight of leaves in contrast to 150 ìg obtained from a litre of *E. coli* culture. The yield of diabody was even higher, i.e. 40 mg per kg of fresh weight of leaves, whereas in *E. coli* the diabodies were expressed to the extent of 50 ìg per litre of bacterial culture.

As regards the chimeric anti-hCG antibody, the chimeric light (mouse V_L domain and human kappa domain) and the chimeric heavy (mouse V_H domain and the human IgG1 domain) chains were expressed in tobacco leaves when the *Agrobacteria* cultures carrying the respective constructs were vacuum-infiltrated individually, as is evident from Figure 3. However these chains did not bind hCG. When these were co-infiltrated, fully functional chimeric antibodies efficiently binding hCG were generated, as seen from the data in Figure 4. The plant leaves therefore have the capability to assemble the full heavy and light chains of an antibody, in a functional way.

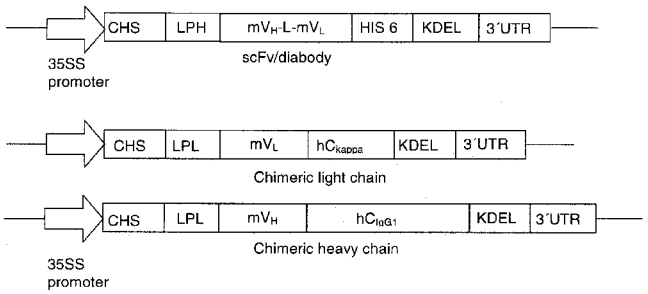

Figure 1. pSSHI plant expression vector constructs for the anti-hCG recombinant antibodies. The single chain variable fragment (scFv) and diabody genes were cloned downstream of the cauliflower mosaic virus 35S enhanced promoter (35SS promoter), the 5′ untranslated region (UTR) of chalcone synthase (CHS), the plant codon optimized leader peptide (LPH) of heavy chain of murine antibody mAb24 (ref. 22), and upstream of the his-6 tag for detection and purification. KDEL is the signal for endoplasmic reticulum retention and the 3′ UTR is from the tobacco mosaic virus. The chimeric heavy and light chains were similarly cloned in the pSSH1 vector, except that the leader peptide (LPL) was from the light chain of the murine mAb24.

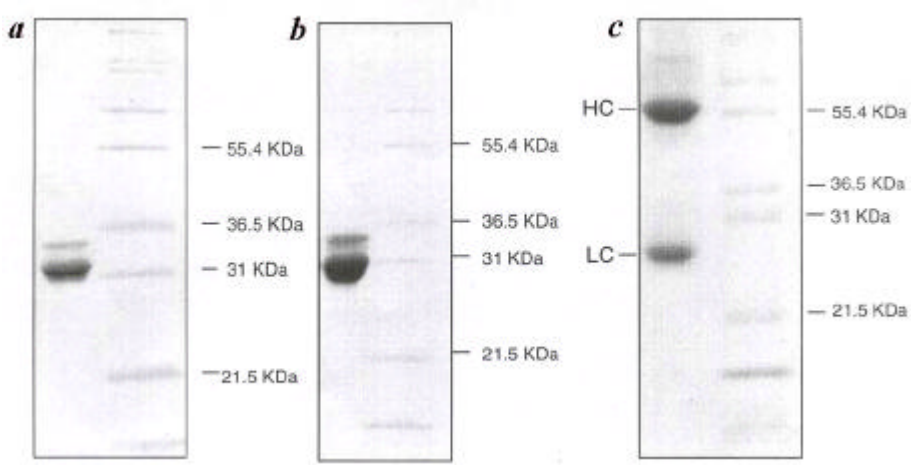

Figure 2. Affinity-purified plant-expressed recombinant anti-hCG antibodies. The recombinant antibodies were purified from tobacco leaves, vacuum-infiltrated with *Agrobacterium* carrying the vector constructs for single chain variable fragment (scFv), diabody and co-infiltration of chimeric heavy chain (HC) and chimeric light chain (LC). The his-6-tagged scFv (***a***) and diabody (***b***) proteins were purified using a Ni-NTA matrix, which gave essentially pure antibody fragments. The minor second bands are due to differential degree of glycosylation of these fragments. The chimeric antibody (***c***) was affinity purified using Protein-A matrix. Note the major band of the heavy and light chains of the immunoglobulin obtained by SDS–PAGE.

The plant-expressed scFv, diabody and chimeric antibodies all bound to hCG in immunoassays. It was however important to determine whether such antigen-binding moieties can effectively inhibit the bioactivity of hCG. This was determined by studying the ability of the antibodies to prevent the binding of hCG to its receptors on target tissues. hCG binds to its receptors with fairly high affinity ($Ka = 10^9\ M^{-1}$), and unless the antibody has equivalent or higher affinity, the binding of hCG to its receptor will not be inhibited. Data in Figure 5 show that all three antigen-binding molecules produced in plants successfully inhibited the binding of hCG to Leydig cell receptors. The inhibitory capacity however differed. The full size chimeric antibody is most effective with an ID_{50} of 10^{-10} M. The scFv and diabody, though effective, required more than 100 times higher concentration. The binding of hormone to its receptor is the first critical step for its action on the cell. The fact that the inhibition of this step leads also to blockage of the subsequent effects, namely hCG-induced testosterone synthesis, has been reported elsewhere[21].

Data reported in this communication clearly demonstrate the ability of plant leaves to express antigen-binding fragments like scFv and diabodies. In contrast to bacterial systems, plants expressed these rAbs with much higher yield. Plants also express the full heavy and light chains, which are not made in prokaryotic expression systems. The light chain expressed individually is unable to bind the antigen, in spite of the presence of complementarity determining regions (CDRs) in the molecule. The same is the case with the heavy chain, though this chain at high concentrations has the property of binding the antigen to a small extent. The full function of binding efficiently emerges by the combination of heavy and light chains. This becomes possible when *Agrobacteria* containing both constructs are co-infiltrated into plant leaves. The combination of heavy and light chains demands the creation of three disulphide bonds, which is accurately achieved in these otherwise mammalian proteins, by plant cells. The yield of pure chimeric antibody was 20–24 mg per kg of fresh weight of leaves. Thus plants can be a cost-effective source of such recombinant antibodies. Preliminary investigations also show that these antibodies can be expressed in non-tobacco plant leaves, like spinach and brinjal. However, further studies are required to identify non-tobacco plants giving equally high, if not better yields.

The three types of recombinant antigen-binding fragments expressed in plants retain their ability to bind hCG. The avidity of binding however differed, with the chimeric antibody having the highest affinity amongst the three. The diabody was nearly as efficient as the scFv, though somewhat better. The three variant forms of the antibody molecule have different applications. The full antibody does not normally penetrate all cells, except those having Fc receptors. Studies have shown that smaller antigen-binding fragments (60–100 kDa) have greater tumour penetration ability than the full size antibodies (150 kDa)[26–28]. Pharmacokinetic studies have demonstrated that these have different clearance rates.

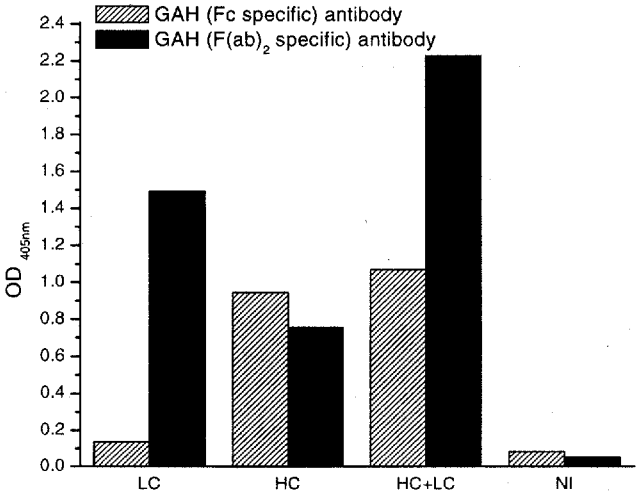

Figure 3. Individual expression, as proteins, of light and heavy chains in plant leaves. The extract obtained from leaves infiltrated with *Agrobacterium* carrying the individual chimeric heavy and light chains or co-infiltration of the two cultures, was used in capture ELISA (described in the text). The light chain (LC) was captured efficiently only by the antibody specific to human $F(ab)_2$ fragment. On the other hand, the heavy chain (HC) bound antibodies are specific to the human Fc region and the $F(ab)_2$ fragment. The heavy and light chains (HC +LC) in extracts obtained from leaves co-infiltrated with the two constructs, bound successfully to both the antibodies. The non-infiltrated (NI) leaf extract was used as control.

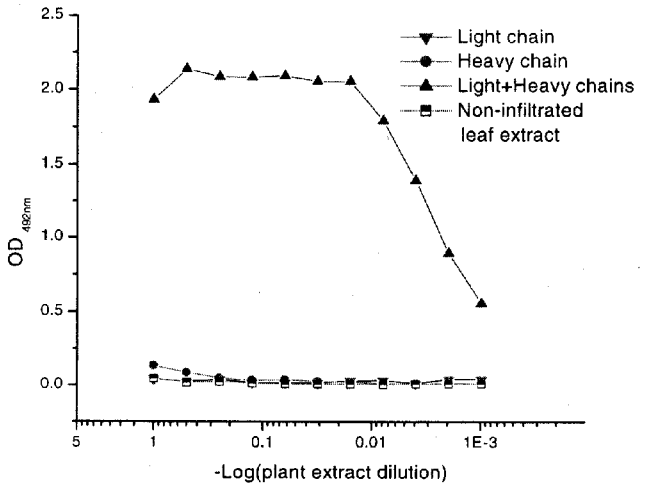

Figure 4. hCG binding ability of transiently expressed chimeric heavy and light chains. The chimeric heavy (HC) and light (LC) chain constructs were infiltrated individually or together into tobacco leaves. Extracts prepared from HC, LC, HC + LC and non-infiltrated tobacco leaves were tested for hCG binding in ELISA. The heavy chain alone, at high concentrations, binds hCG to some extent; but the leaf extract prepared from leaves co-infiltrated with HC- and LC-containing *Agrobacterium*, showed significant binding to hCG. Thus both the heavy and light chains are required for efficient hCG-binding activity.

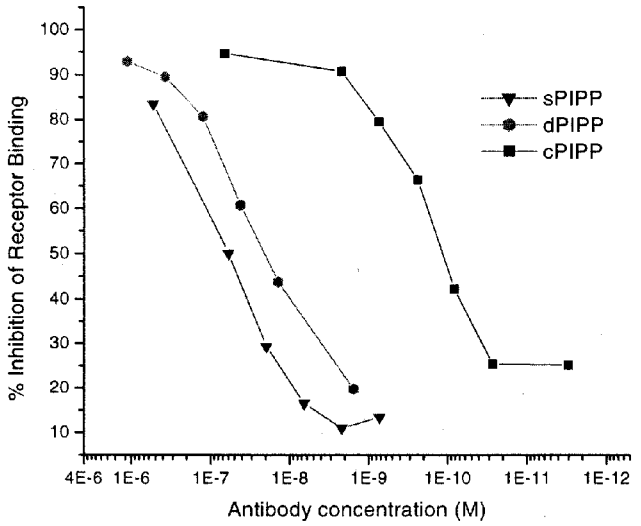

Figure 5. Inhibition of binding of hCG to receptors on Leydig cells. Testicular receptors prepared from Wistar rats were incubated with ^{125}I-labelled hCG in absence or presence of varying concentrations of the plant-expressed rAbs. The percentage of inhibition was calculated with respect to maximal binding of labelled hCG to the receptor preparation. rAbs inhibited the binding of hCG to receptors in a dose-dependent manner. sPIPP and dPIPP showed 50% inhibition at 5.7×10^{-8}M and 2.1×10^{-8} M concentration respectively, whereas cPIPP was able to inhibit at a lower concentration of 1.1×10^{-10} M.

The smaller fragments, scFvs and diabodies, have shorter half life and therefore are advantageous for *in vivo* diagnostic use as well for targeting radioisotopes to tumours, for therapy.

Plant-produced antibodies are well tolerated by humans and have been found effective in humans for preventing oral colonization of *Streptococcus mutans*[29]. Another plantibody has been reportedly effective in preventing vaginal herpes simplex virus (HSV-2) infection in mice[30]. Recombinant antibodies can be successfully folded and expressed in plants and have characteristics similar to those made by hybridomas or mammalian cell cultures[30,31]. Expression in plants provides a suitable way for large-scale expression of antibodies at low cost and are free from contaminants such as endotoxins and animal viruses, possible in case of production in bacterial or animal cells.

Plant-produced anti-hCG antibodies have several potential applications. On account of their low cost these can be used in immunodiagnostic kits for pregnancy and for detection and monitoring of hCG synthesizing cancers. A large number of cancers have been described which make hCG ectopically[6,7,32–34]. Thus the detection of hCG in non-pregnant states would be indicative of such malignancies. Follow-up measurements can be useful for assessing complete surgery and/or therapy by appropriate products. The reappearance of hCG would be indicative of metastasis. The use of antibodies against alpha or beta subunits for therapy of tumours needs further systematic studies. Antibodies against alpha subunit inhibited effectively the growth of human Chago lung cancer cells, in culture and prevented, by passive administration of antibodies, the uptake of the tumour in nude mice[35]. The recombinant anti-hCG antibodies therefore have potential use in diagnosis and therapy of hCG producing tumours, besides their obvious use for emergency contraception and for preventing pregnancy. While chimeric anti-hCG antibodies would be permitted for cancers, further humanization of the chimeric antibodies would be necessary for their use in control of fertility.

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