Production of a Diagnostic Monoclonal Antibody in Perennial Alfalfa Plants

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Abstract: The increasing use of monoclonal antibodies (mAbs) in diagnostic reagents necessitates efficient and cost-effective mAb production methods. In blood banks, one of the most routinely used reagents is the anti-human IgG reagent used for the detection of non-agglutinating antibodies. Here we report the production of a functional, purified anti-human IgG, through the expression of its encoding genes in perennial transgenic alfalfa. Transgenic plants expressing the light- and heavy-chain encoding mRNAs were obtained, and plants from crosses were found to express fully assembled C5-1. The purification procedure yielded mainly the H\textsubscript{2}L\textsubscript{2} form with specificity and affinity identical to those of hybridoma-derived C5-1. The ability to accumulate the antibody was maintained both in parental F\textsubscript{1} lines during repeated harvesting and in clonal material; the antibody was stable in the drying hay as in extracts made in pure water. Also, plant and hybridoma-derived C5-1 had similar in vivo half-lives in mice. These results indicate that plant C5-1 could be used in a diagnostic reagent as effectively as hybridoma-derived C5-1, and demonstrates the usefulness of perennial systems for the cost-effective, stable, and reliable production of large amounts of mAbs. © 1999 John Wiley & Sons, Inc. Biotechnol Bioeng 64: 135–143, 1999.

Keywords: monoclonal antibodies; transgenic alfalfa; protein stability

INTRODUCTION

Monoclonal antibodies are complex, heteromultimeric glycoproteins of wide interest to the biotechnology community. These molecules are used as research reagents in the diagnosis and therapy of a number of important human diseases (Scheinberg and Chapman, 1995), for immunoaffinity purification (Hill, 1995), and as abzymes (Persidis, 1997). As a result, the demand for monoclonal antibodies has increased substantially. Up to now, the use of hybridoma systems has satisfied much of this demand. However, this technology presents some disadvantages, of which the most important are high production costs (Wongsamuth and Doran, 1997) and instability of murine hybridomas in long-term cultures (Coco-Martin et al., 1992; Merrit and Palsson, 1993).

The isolation of cDNA clones encoding the light and heavy chains of antibodies has allowed the expression of antibody genes in various heterologous systems (Wang et al., 1995; Wright et al., 1992). Among these, plants appear to be one of the most promising for cost-efficiency and biosafety, especially for therapeutic applications. Plant viruses are not known to infect humans or animals, and potentially pathogenic human and animal viruses are not capable of replicating in plant cells (Miele, 1997). Biosafety concerns for plantibodies are related to the presence of pesticides, herbicides, and contaminants, such as mycotoxins.

Annual plants, such as tobacco and Arabidopsis, are currently used for the production of antibodies (De Neve et al., 1993; Düring et al., 1990; Fiedler and Conrad, 1995; Hiatt et al., 1989; Ma et al., 1994; Ma et al., 1995). Although these plants are considered to be valuable model systems for testing the feasibility of antibody production in plant cells, some disadvantages could limit their use for large-scale production in agricultural systems. One of these is the obligation to maintain a production seed bank from transgenic lines. This bank would probably need to be reamplified periodically, because most seeds cannot be stored indefinitely (Miele, 1997). Moreover, segregation of genes during sexual reproduction could lead to large variations in antibody production in successive generations (De Neve et al., 1998; Wongsamuth and Doran, 1997). Hence, it becomes important to select new crop plants for antibody production that not only present the common advantages of plant systems (low-cost production of biomass, the ease in scale-up, established practices for efficient harvesting, transporting, storing, and processing) (Whitelam et al., 1993), but also meet the genetic stability requirement, which is essential to insure that the same product is obtained consistently (Miele, 1997). Some living forms, among which many plant spe-
cies, are described as perennial; that is, they remain alive indefinitely. From a biotechnology perspective, these organisms possess one ideal characteristic, the potential to become stable, eternal sources of recombinant molecules. However, because not all plant parts in a perennial plant have perennial characteristics, and it has been proven repeatedly that even constitutive expression is dependent of type, age, or development stage of a tissue in transgenic organisms, it is important to demonstrate that perennial transgenic organisms can be used as more permanent sources of recombinant molecules than short-lived ones.

The model organism that was chosen for the current study is *Medicago sativa* (alfalfa); a perennial legume forage crop; it was chosen not only for its perenniality, but also for its ability to produce high yields without nitrogen fertilization. The model molecule (C5-1) that was chosen is a high-affinity, anti-human IgG monoclonal antibody secreted by a murine hybridoma cell line, and which is suitable for use in anti-human globulin (AHG) reagents. These reagents are widely used in blood banks for phenotyping and cross-matching red blood cells of receivers and donors. C5-1 is a component of an AHG, which allows the detection of incomplete blood group antibodies in human serum (St. Laurent et al., 1993). This antibody is usually produced by large-scale culture of B-cell hybridomas. While reliable, this process is costly, one gram of C5-1 antibody being estimated to ca. $5,000. In comparison with other diagnostic applications of monoclonal antibodies, the market for reagents used in blood bank testing is highly competitive; consequently, prices for reagents are relatively low and the cost-efficiency of antibody production has become a critical issue.

The current study was undertaken to evaluate the potential of perennial organisms, like alfalfa, to produce basically eternal and stable sources of recombinant antibodies.

**MATERIALS AND METHODS**

**Selection of C5-1 Hybridoma Cell Lines**

Hybridoma cell lines were prepared by fusion of SP2/0 mouse myeloma cells with spleen cells of Balb/c mice hyperimmunized with human IgG. The C5-1 hybridoma cell line was screened on the basis of reactivity of supernatent against Rh(D)-positive red blood cells of donors. C5-1 is a component of an AHG, which allows the detection of incomplete blood group antibodies in human serum (St. Laurent et al., 1993). This antibody is usually produced by large-scale culture of B-cell hybridomas. While reliable, this process is costly, one gram of C5-1 antibody being estimated to ca. $5,000. In comparison with other diagnostic applications of monoclonal antibodies, the market for reagents used in blood bank testing is highly competitive; consequently, prices for reagents are relatively low and the cost-efficiency of antibody production has become a critical issue.

The current study was undertaken to evaluate the potential of perennial organisms, like alfalfa, to produce basically eternal and stable sources of recombinant antibodies.

**cDNAs Isolation, Sub-Cloning, and DNA Constructs**

The full-length cDNAs, including the 5’ murine leader sequences, of the heavy (H) and light (L) chains were cloned from a library of hybridoma cell cDNAs as described by Bazin et al. (1992). To add compatible restriction sites for subsequent cloning in the plant expression vector pGA643 (An et al., 1988), they were both sub-cloned into the EcoR I site of pGEM-7Z (Promega, Madison, WI, USA) and excised with *HindIII/XbaI* for L cDNA and *ClaI/XbaI* for H cDNA. These modified fragments were cloned into the binary expression vector pGA643 to generate pGA643-kappa and pGA643-gamma. In these plasmids, each cDNA was inserted between the 35S promoter and the T-DNA transcript 7 gene terminator.

**Plant Transformation, Selection, and Intercrossing**

pGA643-gamma and pGA643-kappa were introduced into *Agrobacterium tumefaciens* LBA4404 by the freeze–thaw method (An et al., 1988). Plant transformation and regeneration were performed as described previously by Desgagnés et al. (1995) using genotype 11.9. Regenerated plants were screened for expression of immunoglobulin chains using northern blots as described below. Transgenic plants expressing the mRNAs for the L and H chains were intercrossed without emasculation. The F1 progeny (plants resulting from sowing seeds obtained from crosses between H and L transgenic lines) was screened by northern hybridization, and transgenic lines coexpressing H and L chains were used for further analysis (Western blotting, ELISA, antibody purification).

**RNA Isolation and Northern Blot Analyses**

Total RNA was isolated from control and transgenic alfalfa leaves as described by de Vries et al. (1988). Total RNA (15 µg/lane) was fractionated on 1% agarose-formaldehyde gels and transferred to Hybond-N nylon membranes. Hybridizations were performed using 32P-labeled probes consisting of a 0.6 kb fragment from the kappa cDNA or the entire gamma chain cDNA for the screening of parental plants. For the screening of F1 progeny plants, both probes were used simultaneously during hybridization.

**Western Blot Analysis**

Leaves were homogenized in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 2 mM PMSF. Extracts were separated by SDS-PAGE on 12% acrylamide and electrotransferred onto a nitrocellulose membrane. The first incubation was carried out with a rabbit anti-mouse antibody (H + L) (Jackson ImmunoResearch Laboratories, West Grove, PA) at 4°C. The secondary antibody was a horseradish peroxidase-conjugated goat anti-rabbit (H + L). Blocking of the membrane and detection of conjugated-peroxidase activity was carried out with the BM chemiluminescence kit from Boehringer Mannheim (Laval, Qc, Canada) as described by the manufacturer. Breakage of the disulfide bonds in the recombinant IgG was performed by using 0.4% β-mercaptoethanol with or without 10 mM sodium ascorbate and heating at 100°C for 5 min.

**ELISA**

Two types of ELISA were used in the current study. For the first ELISA procedure, the wells of microplates (Costar)
were coated overnight with human IgG (human immune serum globulin, Miles, Cutter Biologicals, Elkhart, IN) diluted at 5 μg/mL in a carbonate buffer (100 mM, pH 9.6). This type of ELISA was used to estimate the amount of antibody that specifically binds to human IgG. For the second ELISA procedure, a goat anti-mouse IgG1 (Fc specific) antibody was used as capture antibody. Blocking was done with PBS containing 0.25% casein (PBS-casein). For both ELISA procedures, plant extracts were prepared as described for the affinity purification with human IgG as ligand (see below), and applied directly to the coated plates following dilutions in PBS-casein. After incubation, the plates were washed and the binding of C5-1 was revealed using a goat anti-mouse IgG-peroxydase conjugate (H + L) (Jackson Immm. Research Lab). The enzyme conjugate was revealed with the orthophenylene diamine (Gibco BRL) substrate. Optical density at 490 nm was measured on a microplate reader (Dynatech MR 5000, Alexandria, VA). Results obtained from these two types of ELISA were compared, and the most relevant are presented.

**Human IgG-Sepharose Affinity Chromatography of C5-1 from Leaf Extracts**

Twenty g of pooled leaf tissue from four different F1 transgenic lines were homogenized in 100 mL of extraction buffer A (50 mM Tris-base pH 7.4, 150 mM NaCl, 6 mM PMSF). The homogenate was filtered using one layer of Miracloth and centrifuged (20 min, 13,000 rpm). The supernatant was filtered on a Whatman paper and applied to an affinity column prepared by coupling human IgG to CNBr-Sepharose (Sigma, Oakville, Ont., Canada). The column was washed with phosphate-buffered saline (PBS) (10 mM PO4, 150 mM NaCl, pH 7.4), and the antibody was eluted with an acidic glycine buffer (100 mM, pH 2.3). The antibody-containing fractions were neutralized with 0.1M Tris-HCl (pH 7.0) and dialyzed against PBS. The amount of plant-purified C5-1 in fractions and proteins present in extracts was measured as described by Bradford (1976) using immunoglobulin as standard. Yield estimates of this purification process was obtained by dividing the amount of the purified C5-1 mAb by the initial amount of C5-1 in crude extracts. These amounts were determined by ELISA.

**Large-Scale Purification Using Streamline rProtein Matrix**

For large-scale purification of plant C5-1, 300 g of plant material was homogenized in 1.2 L of an extraction buffer containing 50 mM borate and 4M NaCl at pH 9.0. The homogenate was clarified through filtration on a cheese cloth. This homogenate was then loaded directly by upward flow on an expanded bed of Streamline rProtein A matrix (Pharmacia, Baie D’urfé, Qc, Canada). Loading was performed at 4°C at a rate of 7 mL/min. The column was washed with 250 mL of the extraction buffer and eluted with 50 mM sodium citrate, 50 mM sodium phosphate, and 300 mM NaCl at pH 3.0. Fractions (1.5 mL) were recovered, and immediately neutralized with 100 μL of 1.5M Tris at pH 8.8.

**Hemagglutination Assay**

The reactivity of the plant C5-1 mAb was estimated using the spin-tube technique described by Issit (1985). Rh(D)-positive red blood cells were coated with human IgG by incubation with a human anti-Rh(D) reagent and washed with PBS. Forty μL of a 2% (v/v in PBS) suspension of human IgG-coated red blood cells and 40 μL C5-1 mAb (6 μg/mL) were mixed in a glass test tube, incubated for 5 min at room temperature and centrifuged for 20 sec at 500g. The degree of agglutination was estimated visually according to the size of macroscopic agglutinates. A value of 4+ indicates complete agglutination of red blood cells.

**Stability of mAbs in Alfalfa Extracts**

Leaf material from F1 transgenic lines producing the C5-1 antibody were homogenized in distilled water, and the extracts were brought to neutrality by adding 25 mg NaOH per g leaf fresh-weight during homogenization (neutralized water extracts). The extracts were then left at room temperature. Aliquots were picked during 2 h following extraction and clarified by centrifugation (10 min, 13,000 rpm). Recovery of functional C5-1 in the extracts was measured by ELISA as described above. In a second experiment, hybrid-oma C5-1 was incorporated into neutralized water extracts from nontransformed plants from genotype 11.9 and *Nicotiana tabacum*. Susceptibility of C5-1 antibody to proteolytic cleavage by alfalfa and tobacco endogenous proteases was estimated by immunodetection following SDS-PAGE and electrotransfer. In a third experiment, C5-1 and two other antibodies, one monoclonal anti-Rh(D) antibody (2SF5) and one polyclonal (human serum globulin), were incorporated into neutralized water extracts from a nontransformed alfalfa plant of genotype 11.9. Recovery of C5-1, 2SF5, and human serum globulin from the extracts was measured over 5 d at room temperature using the ELISA.

**Stability of C5-1 in the Source Material Following Harvest**

The aerial part of one F1 transgenic line expressing known levels of C5-1 was cut, and allowed to dry at room temperature and 25% relative humidity. Equal amounts of leaf material were picked at 0 h, 7 h, 1, 2, and 5 d. Susceptibility of C5-1 to degradation during these periods was estimated by Western blots. In a second experiment, one transgenic line was propagated through stem cuttings and transplanted to an adjacent trial field. Aerial material was harvested at 10% flowering and allowed to dry partially for a day in the field, and then dried at 37°C for a week under ventilation. Recovery of functional C5-1 was determined 12 weeks following harvest by ELISA.
Stability of C5-1 Accumulation in Propagated Material

Two F1 transgenic lines were propagated through stem cuttings. For each line, 15 cuttings were allowed to form root material and submitted to two repeated harvests. The third regrowth was collected, and C5-1 accumulation was measured in each clonal propagule.

In Vivo Stability of Plant C5-1

Evaluation of antibody stability was performed as described by Bazin et al. (1994) with some modifications. Briefly, groups of 3 Balb/c mice were injected intraperitoneally with 200 μL of a saline (150 mM NaCl) solution containing 400 μg/mL of C5-1 mAb purified by affinity chromatography either from hybridoma culture supernatent or alfalfa extracts. Serum was collected at various intervals by retro-orbital bleeding, and the concentration of C5-1 mAb was determined in standard ELISA using microplates coated with human IgG.

The ELISA was performed as described above using serial dilutions (1:500–1:8000) of the sera. MAbs were calculated using a standard curve (0–2 ng) prepared with calibrated purified hybridoma-derived C5-1 mAb.

Antigen-Antibody Affinity

Dissociation constants of the antigen–antibody complexes were measured at equilibrium as described by Friguet et al. (1985) using the ELISA procedure described above.

RESULTS

Isolation of a Hybridoma Cell Line Secreting a High-Affinity mAb

Hybridoma cell lines were screened on the basis of reactivity of the supernatant against Rh(D)-positive red blood cells. One of these lines, C5-1, was shown to secrete an anti-human IgG (kappa) mAb (St. Laurent et al., 1993). The C5-1 mAb gave a reactivity similar to a commercial rabbit polyclonal antihuman globulin reagent. The cDNAs of the H and light L chains were cloned from a cDNA library and sequenced.

Integration and Expression of Transgenes in Alfalfa

The cDNAs encoding the light and heavy chains of C5-1 were transferred into alfalfa plants using Agrobacterium-mediated transformation. A total of 15 and 25 in vitro transgenic plants were obtained for constructs with the L and H chains, respectively. Northern blot was used to detect the expression of the heavy- and light-chain genes in 8-L and 5-H transgenic plants grown under greenhouse conditions.

Figures 1A and B show the hybridization signals from these initial transgenic lines. Different crosses between these transgenic lines were designed and performed manually without emasculation. The choice of transgenic lines used for different crosses was based on mRNA level of antibody chains; crosses were performed between both high- and low-expressing lines. Some of these crosses were successful and produced up to five seeds, some remained sterile. Seeds from each different successful cross were sown, the resulting plants were grown under greenhouse conditions, and subsequently tested for mRNA expression using L and H probes simultaneously during hybridization. Figure 1C presents an example of data obtained from two different crosses: (1) involved H chain plant number 4 (Fig. 1B) as pollen donor against L-chain plant number 4 (Fig. 1A); (2) involved H-chain plant number 4 (Fig. 1B) as pollen donor against L-chain plant number 8 (Fig. 1A). Two, out of four plants from cross 1 (Fig. 1C, lanes 1 and 2) were found to express both chains simultaneously; the other progeny plants from cross 1 expressed H chain alone (Fig. 1C, lanes 3 and 4). For cross 2, one progeny plant expressed H chain alone (Fig. 1C, lane 5), one plant expressed L chain alone (Fig. 1C, lane 7), and no signal was detected for the third progeny plant. A total of 7 out of 29 F1 plants from different crosses analyzed were found to coexpress the H- and L-chain mRNAs (full data not shown). Because the progeny

Figure 1. Northern blot analysis of transgenic alfalfa plants. (A) Extracts from plants transformed with pGA643-kappa. Lanes 1, 2, and 4–9 contain RNA extracts from transgenics; lane 3 contains RNA from untransformed plant. (B) Extracts from plants transformed with pGA643-gamma. Lanes 1–5 contain RNA extracts from transgenics; lane 6 contains RNA from untransformed plant. (C) Extracts from plants of the F1 progeny hybridized with a mixture of both the kappa and gamma probes.
plants tested do not result from the same cross, these data were not used to determine segregation ratios of the H-and-L chain genes.

Western blot analysis of protein extracts from F1 progeny plants was used to confirm the expression of light and heavy chains as well as assembled antibody. Results obtained under nonreducing conditions (Fig. 2A) showed that (1) parental L-chain plant (Fig. 1A, lane 4) did not accumulate significant amounts of the corresponding peptide (Fig. 2A, lane L), (2) parental H-chain plant (Fig. 2B, lane 2) contained solely a dimer of H chains (Fig. 2A, lane H), and (3) the two F1 progeny plants (Fig. 2A, lanes F1-a and F1-b) obtained from a cross between these two parental plants contained a strong band which co-migrated with the fully assembled murine C5-1 mAb. Additional bands were also detected in these latter extracts: they are probably assembly intermediates between L and H chains, because their apparent mobility relative to molecular weight standards correspond to the molecular weights of the H\textsubscript{2}L, H\textsubscript{2}, HL complexes (Fig. 2A, lanes F1-a and F1-b). When samples were brought to 0.4% β-mercaptoethanol and heated at 100°C to break the disulfide bonds, a precipitate was formed, and H-and L-chain peptides became undetectable from soluble proteins (Fig. 2B, lane 1). However, when ascorbate was added, the two constitutive subunits remained in solution and were separated on SDS-acrylamide gels (Fig. 2B, lane 2). It was previously shown that the use of sodium ascorbate is helpful for separation of proteins from green tissues of higher plants because it prevents the oxidation of proteins by quinones and other oxidants (Cremer and Van de Wale, 1985). Furthermore, some monoclonal antibodies are sensitive to oxidation, and adding antioxidants to control degradation of these antibodies has been recommended (Birch et al., 1995). Separation in fully denaturing conditions showed that the constitutive subunits of plant-derived C5-1 are identical in size to those of hybridoma-derived C5-1 (Fig. 2B, lane 3).

Quantitative measurements obtained from both ELISA procedures and immunoblots (data not shown) using leaf extracts (picked from 5-week-old shoots) from the seven individual F1 transgenic plants indicated that the level of C5-1 antibody ranged from 0.13% for F1 plants (two plants) obtained by crossing L-chain plant number 9 (Fig. 1A) with H-chain plant number 4 (Fig. 1B), to 1.0% total soluble protein for plants (the two plants used in Western blot analysis) obtained by crossing L-chain plant number 4 (Fig. 1A) as pollen donor against H-chain plant number 2 (Fig. 1B).

In a previous study (Voss et al., 1995), it was shown that the accumulation of fully assembled antibodies and expression levels are regulated by the relative abundance of heavy-chain transcripts. It is probable that the same regulation determined the expression levels obtained in the current study. The two plants (Fig. 1C, lanes 1 and 2) obtained from crosses involving L-chain plant number 4 (Fig. 1A) against H-chain plant number 4 (Fig. 1B) have an expression level of approximately 0.4%; however, the expression level of the antibody was 1% for progeny plant (F1-a) used in Western blot analysis, and resulting from the cross involving the same L-chain plant number 4 (Fig. 1A) against H-chain plant number 2 (Fig. 1B), which expresses the H-chain mRNA at higher levels than H-chain plant number 4 (Fig. 1B). The signal of the H-chain mRNA (data not shown) obtained for this F1 plant was also higher than that of the two plants (Fig. 1C, lanes 1 and 2). Crossing plants with lowest expression levels for L- and H-chain mRNAs resulted in the lowest level of antibody expression (0.13%). This result confirms that the prerequisite for producing functional antibodies in high levels is the high expression of both heavy- and light-chain genes (van Engelen et al., 1994).

**Purification of C5-1 mAb**

Affinity purification against immobilized human IgG was used initially to purify the C5-1 mAb from leaf extracts of transgenic alfalfa plants. SDS-PAGE analysis of the puri-
fied protein under reducing conditions (data not shown) showed that the two chains have the same mobility as their counterparts from hybridoma cells.

For large-scale purification, expanded bed adsorption (STREAMLINE rProtein A) was chosen to allow loading of partially clarified plant extracts containing colloidal material at a high-flow rate. Using this technology, plant C5-1 was purified from non-clarified crude plant extracts mainly as a single peptide as shown by Coomassie staining on SDS-PAGE gels (Fig. 3). Other bands with molecular weights corresponding to the molecular weights of H2L and H2 were present, but barely detectable.

Antigen-Binding and Hemagglutinating Activities

ELISA was used to test the binding capacity of the assembled antibody to human IgG antigen. Protein extracts from plants expressing either heavy- or light-chain peptides individually, were also included in the assays. Results (Table I) showed that extracts from plants expressing the heavy- or light-mRNA chains alone cannot react with human IgGs, and that the extract from F1 progeny plant has a specific activity (optical density per 100 ng antibody) similar to that of C5-1 from hybridoma cells. True affinity of purified plant-derived C5-1 for its antigen was compared to that of hybridoma-derived C5-1 by measuring dissociation constants at equilibrium. KdS were 4.7 × 10^{-10} M and 4.6 × 10^{-10} M for plant- and hybridoma-derived C5-1, respectively.

Hemagglutination tests were used to test whether the plant-produced antibody was able to bind human IgG bivalently. An affinity-purified plant C5-1 was used in parallel with the hybridoma-derived C5-1 mAb. Results (Table II) showed that the plant-derived C5-1 mAb specifically agglutinated anti-D-sensitized human RBC giving a complete (4+) reaction at 6 μg/mL, which is similar to that obtained with hybridoma-derived C5-1 mAb.

Table I. Specific activity (OD per 100 ng) of parental and F1 transgenic plants.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Optical density</th>
<th>Specific activity</th>
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<tbody>
<tr>
<td>IgG from hybridoma cells</td>
<td>0.332 ± 0.032</td>
<td>0.235 ± 0.020</td>
</tr>
<tr>
<td>L (1/1)</td>
<td>0.000</td>
<td>—</td>
</tr>
<tr>
<td>H (1/1)</td>
<td>0.000</td>
<td>—</td>
</tr>
<tr>
<td>HL (F1, 1/32)</td>
<td>0.334 ± 0.011</td>
<td>0.267 ± 0.080</td>
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Stability of mAb in Alfalfa Extracts

Stability of C5-1 in crude extracts was initially demonstrated by homogenization of the leaves of F1 transgenic lines in water. Results show that water alone was as good an extractant for C5-1 as protective buffer A (50 mM Tris-base pH 7.4, 150 mM NaCl, 6 mM PMSF), and that C5-1 remained 100% stable at room temperature for at least 2 h within this minimal extraction system (Table III).

Further testing demonstrated that hybridoma-derived or plant-derived C5-1 are readily insolubilized in tobacco extracts (Fig. 4A). Incubation of different antibodies, including C5-1, in extracts from nontransformed genotype 11.9 demonstrated that not all mAbs would be equally stable for extended periods (Table IV); however, recovery of the three antibodies was 100% after 6 h incubation in water extracts at room temperature.

Stability of C5-1 Accumulation in Propagated Material

There was no apparent loss in the ability to synthesize and accumulate fully functional recombinant C5-1 in plants produced through stem propagation (Table V).

Stability of C5-1 in the Source Material Following Harvest

Results show that C5-1 is stable in drying hay (Fig. 4B). There was no apparent degradation during the trial when the hay was allowed to dry in controlled conditions. When the plants were grown in normal field conditions, they accumulated C5-1 at higher rates than greenhouse plants (140 μg/g dry hay) and the ELISA showed that the functional protein is stable in dry hay within 12 weeks of harvest.

In Vivo Stability of Plant C5-1

The plant-derived C5-1 was shown to be as stable as hybridoma-derived C5-1 in the blood stream of mice follow-
ing intramuscular injection (Fig. 4C). The stability of plant C5-1 is probably due to the plant conferred glycosylation.

### DISCUSSION

The studies described herein demonstrate that transgenic alfalfa is a viable source of monoclonal antibodies for diagnostic use. Although the production of antibodies in plants is not new, this is the first demonstration of the production of such a complex multimeric protein in a perennial plant. The antibody produced herein (C5-1) is a component of an AHG reagent used in blood banks for the detection of incomplete blood group antibodies. The strategy used for integration of antibody-chain genes and generation of transgenic plants coexpressing H- and L-chain mRNAs was similar to that described by Hiatt et al. (1989). Native signal peptides were neither removed nor replaced by other signal peptides of plant origin, as previously described by Dürring et al. (1990). In this latter study, the attempt to replace the native signal of the murine antibody with a barley α-amylase signal sequence led to unexpected mistargeting of the immunoglobulin chains to the chloroplast.

Although it was previously reported that the CaMV35S promoter did not function in alfalfa at rates comparable to those in tobacco or other model species (Navarez-Vasquez et al., 1992), transgenic alfalfa plants expressing the C5-1 antibody at levels between 0.13 and 1.0% total soluble protein were obtained in this study following sexual cross. These levels were estimated by two ELISA procedures that gave nearly equivalent results by immunoblot in which known quantities of purified C5-1 from hybridoma culture were loaded (data not shown). Comparable levels of antibody expression have been previously reported for tobacco and *Arabidopsis*, (De Neve et al., 1993; Hiatt et al., 1989; van Engelen et al., 1994).

Purification of alfalfa C5-1 was easily performed through affinity chromatography using human IgG. There was no apparent loss of C5-1 during the purification process, and thus, the yield from in planta C5-1 to purified C5-1 was estimated to be more than 70%. The plants used for purification of C5-1 were heterogeneous with regard to the expression level of the C5-1 antibody. Thus, they were not intended to be representative of transgenic lines that would be used for commercial production. The main objective was to obtain transgenic alfalfa plants that could produce detectable amounts of diagnostic monoclonal antibodies with the same functional characteristics needed for its end use, hence, confirming the feasibility of this new approach.

Although several probable assembly types were detected in crude extracts following Western blotting, the large-scale purification procedure using STREAMLINE rProtein A yielded mainly the $H_2L_2$ form. The other probable assembly intermediates and heavy chains were present in very low frequency.

**Table III.** Stability of plant C5-1 in crude protein extracts.

<table>
<thead>
<tr>
<th>Time after extraction</th>
<th>Total proteins (mg/mL)</th>
<th>C5-1* (µg/mL)</th>
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<tbody>
<tr>
<td>Control^b</td>
<td>2.21</td>
<td>2.40</td>
</tr>
<tr>
<td>0 h</td>
<td>2.47</td>
<td>2.45</td>
</tr>
<tr>
<td>1 h</td>
<td>2.32</td>
<td>2.45</td>
</tr>
<tr>
<td>2 h</td>
<td>2.24</td>
<td>2.62</td>
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* Determined by ELISA as described in Materials and Methods.

^ Control extract in buffer A (see Experimental protocol).
levels, and barely detectable with Coomassie staining. Their signals represent less than 5% the signal obtained for the H₂L₂ form. The immunoreactivity of purified C5-1 was signals represent less than 5% the signal obtained for the H₂L₂ form. The immunoreactivity of purified C5-1 was similar to its counterpart from hybridoma cells when tested in ELISA and standardized hemagglutination assays. The purified C5-1 mAb gave reactivity responses and antigen- antibody produced in the current study also shows remarkable stability in the plant, even when harvest and during were performed under current cultural practices in open-field conditions. Perenniality of the transgenic character was shown to be equal to that of the plant, that is, (1) the accumulation of C5-1 remained stable in F1 lines following repeated harvesting over 2.5 years (2) the accumulation of C5-1 was essentially similar in clonal propagules obtained through stem cuttings than in original F1 lines.

Currently, there are no reports on large-scale purification of mAbs from transgenic plants which describe a methodology that would yield grams of electrophoretically pure mAb at low cost (Whitelam and Cockburn, 1996). In our hands, tenths of milligrams of C5-1 were purified to homogeneity using the simple methodology developed for crude extracts. Scaling-up of the purification process was facilitated by the use of expanded bed adsorption chromatography. This technology produced a purified peptide from a large volume of roughly clarified extracts within a reduced time frame, and thus seems to open new approaches to the reduction of purification costs from colloidal extracts. The results of this study bring other perspectives to previous studies (Kusnati et al., 1997), which dealt with the quantification of purification of recombinant proteins from transgenic plants.

One of the compelling reasons for using plants as hosts for the production of recombinant proteins is the significant cost advantage. Following this study, production costs were estimated for year-long exploitation of an alfalfa system under greenhouse conditions. Including heating, manpower, and consumables for extraction and purification, the cost per g of C5-1 was estimated to $500–$600, in a 250 m² greenhouse compared to $5000 per g when produced with a hybridoma system.

Finally, plant-derived C5-1 exhibits in vivo stability comparable to hybridoma derived C5-1 in the blood stream of mice. This stability is probably due to alfalfa conferred glycosylation. It was previously shown (Winkelhake and Nicolson, 1976) that non-glycosylated antibodies were cleared more rapidly from the circulation than wildtype antibodies. However, this conclusion may not be true for all IgG subclasses because Tao and Morrison (1989) have obtained similar half-lives for normal and non-glycosylated IgG1 antibodies. Although C5-1 is not intended for in vivo use, this result indicates that other alfalfa-derived mAbs could have the in vivo stability required for therapeutic use. Additional experiments are planned to further study this interesting aspect.

References
De Neve M, Van Houdt H, Bruyns, A.-M., Van Montagu M, Depicker,

Table IV. Stability of antibodies coharvested with, and incubated within alfalfa extracts.

<table>
<thead>
<tr>
<th>Time after extraction</th>
<th>C5-1 (µg/mL)</th>
<th>2SF5 (µg/mL)</th>
<th>hISG (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.2 ± 0.9</td>
<td>3.6 ± 0.2</td>
<td>7.7 ± 0.9</td>
</tr>
<tr>
<td>2 h</td>
<td>4.9 ± 0.7</td>
<td>3.9 ± 0.5</td>
<td>7.1 ± 0.8</td>
</tr>
<tr>
<td>6 h</td>
<td>4.1 ± 0.2</td>
<td>4.0 ± 0.5</td>
<td>6.0 ± 0.6</td>
</tr>
<tr>
<td>1 d</td>
<td>4.6 ± 0.5</td>
<td>0.8</td>
<td>5.6 ± 0.3</td>
</tr>
<tr>
<td>4 d</td>
<td>4.3 ± 0.1</td>
<td>0.75 ± 0.03</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>12 d</td>
<td>0.58 ± 0.07</td>
<td>0.18 ± 0.02</td>
<td>1.8 ± 0.1</td>
</tr>
</tbody>
</table>

Table V. Stability of extractable C5-1 in clonal propagules.

<table>
<thead>
<tr>
<th>Expression level (µg/g)</th>
<th>F1 line 2</th>
<th>F1 line 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting material</td>
<td>14.4</td>
<td>14.4</td>
</tr>
<tr>
<td>Average (15 propagules)</td>
<td>17.0 ± 2.5</td>
<td>16.0 ± 1.99</td>
</tr>
<tr>
<td>Highest level</td>
<td>21.8</td>
<td>19.7</td>
</tr>
<tr>
<td>Lowest level</td>
<td>12.7</td>
<td>12.4</td>
</tr>
</tbody>
</table>