Regulatory approval and a first-in-human phase I clinical trial of a monoclonal antibody produced in transgenic tobacco plants

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Summary
Although plant biotechnology has been widely investigated for the production of clinical-grade monoclonal antibodies, no antibody products derived from transgenic plants have yet been approved by pharmaceutical regulators for clinical testing. In the Pharma-Planta project, the HIV-neutralizing human monoclonal antibody 2G12 was expressed in transgenic tobacco (Nicotiana tabacum). The scientific, technical and regulatory demands of good manufacturing practice (GMP) were addressed by comprehensive molecular characterization of the transgene locus, confirmation of genetic and phenotypic stability over several generations of transgenic plants, and by establishing standard operating procedures for the creation of a master seed bank, plant cultivation, harvest, initial processing, downstream processing and purification. The project developed specifications for the plant-derived antibody (P2G12) as an active pharmaceutical ingredient (API) based on (i) the guidelines for the manufacture of monoclonal antibodies in cell culture systems; (ii) the draft European Medicines Agency Points to Consider document on quality requirements for APIs produced in transgenic plants; and (iii) de novo guidelines developed with European national regulators. From the resulting process, a GMP manufacturing authorization was issued by the competent authority in Germany for transgenic plant-derived monoclonal antibodies for use in a phase I clinical evaluation. Following preclinical evaluation and ethical approval, a clinical trial application was accepted by the UK national pharmaceutical regulator. A first-in-human, double-blind, placebo-controlled, randomized, dose-escalation phase I safety study of a single vaginal administration of P2G12 was carried out in healthy female subjects. The successful completion of the clinical trial marks a significant milestone in the commercial development of plant-derived pharmaceutical proteins.

Introduction
The use of plants to produce recombinant pharmaceutical proteins is known as molecular pharming (Ma et al., 2003; Stoger et al., 2014). This niche use of plant biotechnology was initiated in 1989 by the expression of a catalytic monoclonal antibody in tobacco (Nicotiana tabacum) plants (Hiatt et al., 1989) and led to a range of proof-of-principle studies in which pharmaceutically relevant proteins, including various antibody formats, subunit vaccines, autoantigens, hormones, cytokines, blood products, enzymes and technical reagents, were expressed in a diverse range of plant-based production platforms (Ma et al., 2005a). The use of plant biotechnology for the production of pharmaceutical proteins is considered advantageous mainly because of the scalability of production and the potentially low manufacturing costs (Twyman et al., 2005; Sack et al., 2015). Less widely appreciated benefits include the low capital investment and minimal technical expertise required during production, and the transferability of plant biotechnology to resource-poor regions, where local health priorities may differ from those catered for by the global pharmaceutical industry (Ma et al., 2005a, 2013).

Despite enthusiasm in academia, the commercial uptake of molecular pharming has been cautious. In the case of monoclonal antibodies (mAbs), this reflects the existence of a ‘gold standard’ production platform based on mammalian cell cultures that are well established in the industry and compliant with good
manufacturing practice (GMP). In contrast, the regulatory path for plant-made pharmaceuticals (PMPs) has been much less clear. The differences between platforms based on sterile cell cultures and nonsterile whole organisms led to doubts about the quality and consistency of mAbs produced in plants, resulting in an uncertain environment for industry engagement in the development of PMPs (Ma et al., 2005b; Fischer et al., 2012).

In the field of HIV prevention, HIV-neutralizing mAbs have many potential clinical uses, including immunoprophylaxis, in which mAbs are applied topically as microbicides at the site of infection, either in the vagina or rectum (Shattock and Solomon, 2004; Ramessar et al., 2010). However, large (milligrams) doses are required for effective protection, and the product must be used regularly before sexual intercourse, meaning that production capacity would need to exceed 1000 kg/year to meet global demand (Shattock and Moore, 2003). Because the costs must also be kept low, it is unlikely that mAb-based microbicides could be produced using conventional manufacturing approaches, particularly because the greatest need is in developing countries. Plant biotechnology therefore represents an attractive alternative for this application area because of the combination of low cost and scalability.

The mAb 2G12 is one of a small number of known HIV-neutralizing mAbs (Walker et al., 2011) and one of only a handful that has undergone clinical evaluation in humans (Armbuster et al., 2002, 2004; Joos et al., 2006; Mehandru et al., 2004). For previous clinical studies, mAb 2G12 was produced in Chinese hamster ovary (CHO) cells and a comprehensive safety portfolio was accumulated for the antibody in these studies. The established characterization of mAb 2G12 and its history of clinical safety were important reasons for its selection as the lead product in this project.

The specific objectives of the project were to:
1. Identify the key regulatory issues relating to the GMP-compliant production and processing of plant-derived antibodies.
2. Develop a suitable transgenic plant line producing mAb 2G12 (known as P2G12).
3. Develop procedures for plant cultivation and downstream processing to address the key regulatory issues identified above.
4. Establish specifications for plant-derived mAbs acceptable for human use.
5. Design and perform a clinical trial to establish the safety of a plant-derived mAb.

This study describes the development of a manufacturing process that was ultimately accepted by national regulatory authorities in Europe. It is organized according to the guidance provided in the European Medicines Agency (EMA), ‘Guideline on the quality of biological active substances produced by stable transgene expression in higher plants’ (EMEA/CHMP/BWP/48316/2006) that came into effect 1 February 2009 and illustrates the path followed to establish a GMP-compliant process for this novel class of biotechnology-derived pharmaceutical product.

Results
Development genetics
Leaf discs from Nicotiana tabacum cv. Petit Havana cv. SR1 plants were transformed by Agrobacterium tumefaciens strain GV3101 carrying the expression vector pTRAp-2G12-Ds, which contained the 2G12 heavy and light chain genes and the marker gene DsRed. T0 lines were screened for high-level antibody expression and were self-pollinated. The best expressing T1 plants were also self-pollinated, and the process was repeated until the T6 generation.

Characterization of the master and working seed banks
T5 generation seeds derived from a single initial transformation event were designated as the master seed bank (MSB). This plant line was found to be homozygous for the single transgene locus by segregation analysis, which confirmed single-gene Mendelian segregation. The germination rate was >90%.

For genetic characterization and assessment of genetic stability, a genomic Southern blot was carried out on the T5, T6 and T7 generation plants using probes specific for the heavy and light antibody chain genes (Figure S1). Fragments of the correct size for each transgene were detected, and no truncated copies were present. Defined amounts of vector DNA were used as a standard to determine the number of transgene copies present in the genome. The intensity of the hybridization signal was identical in all the tested plants and did not change between the T5 and T7 generations, again confirming Mendelian segregation. The line contained fewer than 10 copies of the antibody genes (two copies estimated for the heavy chain and six copies for the light chain) at a single genetic locus (data not shown).

Northern blot analysis was carried out to confirm the size of the antibody chain transcripts in three or six individual plants from the T5, T6 and T7 generations. Transcripts of the expected size (1751 nt for the heavy chain and 1037 nt for the light chain) were detected using specific probes, and no truncated transcripts were present (Figure S2).

To confirm the identity of the transgene sequences, the coding regions (for the antibody gamma and kappa chains) and immediately adjacent sequences were amplified and sequenced. This analysis was carried out using three individual plants from the MSB (T5) and three individual plants from the subsequent generation (T6). For the heavy chain gene, approximately 180 bp of upstream sequence and a minimum 50 bp of downstream sequence were included. For the light chain gene, 50–150 bp of upstream sequence and 50 bp of downstream sequence were included. The correct sequences were confirmed.

Stability of antibody production in transformed plants
Antibody expression levels were monitored in 20–40 individual plants in each generation. The performance and stability of the introduced trait were confirmed by carrying out a closing comparison of all generations (T1–T4) upstream of the T5 MSB and of T5 and T6 plants derived from the MSB. Antibody accumulation was evaluated along with two additional production-relevant parameters: biomass and plant growth (Figure 1). Ten T1 plants and 20 plants from each subsequent generation were grown simultaneously and the antibody levels were determined using an extract from all harvestable leaves (6–8 per plant). The results showed that the average antibody yield was ~10 µg/g in T1 plants, increasing to ~25 µg/g in T3 plants. Thereafter, the yield was effectively stable. There was no difference in leaf biomass or plant height at harvest across all the generations of plants. Subsequent testing extending to the T10 generation has confirmed this stability data (not shown).

Manufacturing issues
General manufacturing strategy
The general manufacturing strategy shown in Figure 2 represents the first production phase (encompassing plant cultivation,
harvesting and primary processing) and the second production phase (encompassing product isolation and purification). Details are provided in the methods section.

**Batch definition**

A batch of transgenic tobacco plants was defined as plants that were seeded from the same working seed bank (WSB) aliquot on the same day, cultivated under the same environmental conditions, transferred into individual pots on the same working day and harvested on the same working day. The WSB was defined as the T6 generation of seeds derived from T5 plants grown from the MSB.

A batch of purified P2G12 was isolated from the leaves of a batch of transgenic plants according to the process flowchart in Figure 2. A typical batch comprised ~250 kg of tobacco leaves, corresponding to a crude extract volume of ~900 L. This resulted in the yields, leaf biomass and plant height shown in Figure 1. Results represent the means ± SD of 20–40 plants per generation.

**Figure 1** Phenotypic characterization of transgenic plants grown from T1 to T5 seeds. Antibody yield, leaf biomass and plant height are shown. Results represent the means ± SD of 20–40 plants per generation.

**Figure 2** Process flowchart for the production of P2G12 active pharmaceutical ingredient (API). Boxes in pink represent the ‘First production phase’, and boxes in green represent the ‘Second production phase’.

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Control of the drug substance

The drug substance (P2G12) was characterized along with product-related and process-related impurities. Many of the assays are standard within the biopharmaceutical industry, whereas others (including the antibody concentration and activity tests) are product specific and were adopted because similar tests were used for the specification of CHO cell-derived 2G12 (C2G12) in previous clinical trials (Armbruster et al., 2002).

Characterization of the P2G12 drug substance (API)

Four batches of P2G12 were manufactured. Batch #080902 was a preclinical batch specifically prepared for preclinical toxicology studies. Batch #090622 was an API process development batch. Batch #090821 was an API development batch prepared to assess the procedures for processing the API into the investigational medicinal product (IMP). Batch #090918 was the clinical trial batch.

The molecular mass, peptide maps, monosaccharide composition and N-linked glycan structures of two batches (preclinical batch #080902 and clinical batch #090918) were characterized by M-Scan GmbH, Freiburg, Germany. The mass profiles and the calculated masses for intact P2G12, as well as the light and heavy chains released after reduction, were consistent with anticipated results and comparable between the two batches (Figure S3).

On-line liquid chromatography–electrospray ionization mass spectrometry (LC–ESI–MS) analysis of the reduced/carboxymethylated tryptic digest of the two P2G12 batches produced consistent signals, allowing 209 of 214 residues (97.7%) of the light chain and 209 of 214 residues (97.7%) of the heavy chain to be mapped. The same set of peptides was detected in both batches (Figure S4).

Monosaccharide analysis was consistent between the two batches and indicated the presence of equivalent ratios of fucose, xylose, mannose, galactose and N-acetylgalactosamine (GlcNAc), as well as the absence of N-acetylgalactosamine (GalNAc) (Figure S5). For N-glycan analysis, the intact antibody samples were digested with trypsin, lyophilized and then purified after digestion with peptide-N4-(N-acetyl-b-glucosaminy1) asparagine amidase A (PNGaseA). The fraction containing N-linked oligosaccharides was permethylated, and a portion of the sample was analysed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry to obtain molecular weight data. Both samples yielded a major peak at m/z = 1996 consistent with the composition Hex3HexNAcPentFuc. Nanospray mass spectrometry of the permethylated N-glycans yielded a single signal in each sample consistent with the release of HexNAc from nongalactosylated antennae. Linkage analysis of the N-linked oligosaccharides released by PNGase A yielded similar profiles in both batches. The linked monosaccharides we detected were consistent with those expected based on the MALDI and nanospray data. Finally, glycoproteomic analysis was carried out by reverse phase liquid chromatography–electrospray ionization mass spectrometry (RP–LC–ESI–MS). A single predominant glycoform was identified, attached to Asn 297 within the Fc region of the antibody gamma chain. This was a bi-antennary N-glycan with [\(\beta(1,2)\)]-xylose and [\(\alpha(1,3)\)]-fucose attached to the core, with N-acetylgalactosamine residues on both antennae (GnGnXF) (Figure 3).

The API specification and results from the analysis of three batches are presented in Table 1a. The specification was based on that developed for C2G12, which has the same intended use. All results for the three P2G12 batches were within specification, with the exception of the biological activity IC\(_{50}\) measurement for batch #090918 which was 21 μg/mL (the specification was <20 μg/mL).

The Fraunhofer IME manufacturing facility was subject to a GMP inspection and received manufacturing authorization according to the German Medicines Act (Arzneimittelgesetz) in November 2009 for nonsterile monoclonal antibodies from transgenic plants for clinical phase I studies (Licence no: 24.30.12/09-002).

Stability of P2G12 API

Stability testing was carried out on batches #080902, #090622, #090821 and #090918 under a variety of conditions. Samples

![Figure 3](image-url)
were analysed for protein aggregation, monomeric and dimeric antibody content, antibody concentration and HIV-neutralization IC50.

At °C, there was no change in the test substance after 6 months (#090918), 9 months (#090821) or 12 months (#090622). Stability was also demonstrated at °C for at least 3 months (#090622 and #090918), at room temperature for 10 days (#090918 and #080902) and at °C for 6 days (#080902). A significant loss of antibody was only detected when P2G12 was stored at °C (#090622).

**Characterization of P2G12 drug product (IMP)**

The bulk API was sterilized by filtration and aliquotted into sterile containers by McEwen Laboratories Ltd, Pangbourne, UK. The specification for the IMP was based on that for the bulk API and is presented in Table 1b. The results show that the IMP manufacturing process had no impact on the quality of the drug product. The HIV-neutralization IC50 was 11.5 μg/mL.

### Table 1

<table>
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<tr>
<th>Test Description</th>
<th>Specification/Limit</th>
<th>Result (16.2.2011)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Appearance</strong></td>
<td>Clear liquid without visible particulates</td>
<td>Complies</td>
</tr>
<tr>
<td><strong>Labeling</strong></td>
<td>Matches label on file</td>
<td>Complies</td>
</tr>
<tr>
<td><strong>Sterility</strong></td>
<td>Passes</td>
<td>Pass</td>
</tr>
<tr>
<td><strong>Antibody content</strong></td>
<td>22–28 mg/mL</td>
<td>25 mg/mL</td>
</tr>
<tr>
<td><strong>Purity</strong></td>
<td>IgG monomers and dimers &gt;95%</td>
<td>IgG monomers and dimers &gt;98%</td>
</tr>
<tr>
<td><strong>Bioburden</strong></td>
<td>Complies with PhEur 5.1.4 for vaginal use</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

### Table 1b

<table>
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<tr>
<th>Test Method</th>
<th>Specification/Limit</th>
<th>Result (16.2.2011)</th>
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</thead>
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<td><strong>Appearance</strong></td>
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<td>Complies</td>
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<tr>
<td><strong>Protein concentration</strong></td>
<td>22–28 mg/mL</td>
<td>24.0</td>
</tr>
<tr>
<td><strong>Antibody concentration</strong></td>
<td>22–28 mg/mL</td>
<td>24.0</td>
</tr>
<tr>
<td><strong>Purity</strong></td>
<td>IgG monomers and dimers ≥95%</td>
<td>98.8/0.7 – 99.53%</td>
</tr>
<tr>
<td><strong>Purity/identity</strong></td>
<td>Size specific protein</td>
<td>Complies</td>
</tr>
<tr>
<td><strong>Identity</strong></td>
<td>Consistent with amino acid sequence</td>
<td>Complies</td>
</tr>
<tr>
<td><strong>Protein A</strong></td>
<td>&lt;10 ng/mg IgG</td>
<td>0.33 ng/mg</td>
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<tr>
<td><strong>Host cell proteins</strong></td>
<td>&lt;0.1% of total protein content</td>
<td>Complies</td>
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<tr>
<td><strong>DNA concentration</strong></td>
<td>&lt;15 pg/mg IgG</td>
<td>2.8 pg/mg IgG</td>
</tr>
<tr>
<td><strong>Nicotine</strong></td>
<td>&lt;200 ng/mg IgG</td>
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<tr>
<td><strong>Endotoxin</strong></td>
<td>&lt;1.25 EU/mL</td>
<td>n.d.</td>
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<tr>
<td><strong>Biological activity</strong></td>
<td>IC50 for HIV-1 RF strain &lt;20 μg/mL</td>
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</tr>
<tr>
<td><strong>Bioburden</strong></td>
<td>Complies with PhEur 5.1.4 for vaginal use</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
Preclinical toxicity, immunogenicity and pharmacokinetics

A preclinical toxicity and local irritancy study of P2G12 was carried out in New Zealand white rabbits after a single intravaginal dose, using a protocol which mimicked the same simple formulation of antibody in saline and the proposed clinical protocol. There were no treatment-related dose observations or clinical signs, and no animals died prematurely. Specifically, there was no evidence of vaginal irritation following dose administration, and none of the investigations, including postmortem organ weights, macropathology and histopathology, revealed any treatment-related changes. The immunogenicity of P2G12 was assessed by testing vaginal fluid and serum. No specific responses were observed against the antibody protein backbone or the plant N-glycans associated with the antibody (data not shown).

Preliminary pharmacokinetic characteristics were also measured in the rabbits following the single-dose intravaginal administration of P2G12. The analysis of intravaginal fluid by P2G12-specific enzyme-linked immunosorbent assay (ELISA) revealed the presence of P2G12 in all five rabbits after 24 h, and the mAb was still detected in four of the remaining five rabbits after 8 days. In the same study, the testing of serum samples using the same ELISA revealed that no P2G12 was detected on days 2 or 8 and remained above the lower limit of quantitation (15 ng/mL).

Clinical trial authorization and ethical approval of the phase I clinical trial

The clinical trial protocol and informed consent form were approved by an independent ethics committee in April 2010. The request for a clinical trial authorization (CTA) was accepted by the UK Medicines and Healthcare products Regulatory Agency (MHRA) in April 2011, allowing the clinical trial to commence (Eudract No. 2009-015609-38). The trial was completed in November 2011. Thirty-five volunteers were screened for entry into the study, and 11 subjects were selected and randomized. The schedule for the trial is summarized in Table 2. All 11 subjects completed the study, and there were no major study protocol deviations. No major safety issues were identified during the study.

An adverse event was defined as any untoward medical occurrence in a clinical investigation subject administered an investigational product at any application level, although this did not necessarily involve a causal relationship with the treatment. There were 33 adverse events reported during the study, but none were classified as serious, and the majority affected the placebo group. Twelve were of moderate severity, and 21 were classified as mild. Five patients reported adverse events that were potentially related to the clinical intervention, primarily because of the temporal relationship between dosing and the appearance of symptoms. Of these, three received P2G12. One subject reported mild intermittent vaginal discomfort starting 24 h after dosing (14 mg P2G12 group) and lasting for 24 h. Another reported mild intermittent vaginal itching starting 10 days after dosing (28 mg P2G12 group) which lasted for 5 days. The last subject reported mild thirst from 1 h after dosing (28 mg P2G12 group) for several hours. There were no adverse events related to changes in laboratory results, vital signs or general physical examination. Overall, P2G12 was well tolerated and safe.

There were no specific anti-P2G12 immunological changes detected in serum (at 28–35 days) or vaginal fluid (at 72 h) following intravaginal administration of P2G12 at any dose level. The studies separately investigated antibody responses to the protein backbone of P2G12 and the plant-derived N-type glycans. There was some evidence for pre-existing serum IgM and IgA reactivity to P2G12 in the predose samples (data not shown).

Serum analysis did not demonstrate any evidence that P2G12 was systemically absorbed at any time point, following administration at any of the doses. Vaginal fluid samples intended for the analysis of P2G12 concentrations were compromised during transportation and were thawed on arrival at the investigating laboratory, possibly for up to 24 h. Because no further vaginal fluid samples were available, the assays were carried out as planned. Despite the potential for proteolytic degradation in transit (although the freezing buffer contains a cocktail of antiproteolytic reagents to prevent this), P2G12 was still detectable in samples taken up to 8 h after administration (data not shown).

Discussion

Passive immunization and antimicrobial products are two application areas that could benefit significantly from new manufacturing technologies for mAbs, and it is easy to envisage new applications and the increasing use of mAbs once the constraints of cost and scalability are addressed (Paul et al., 2013). The use of HIV-neutralizing antibodies as microbicides have been considered for many years (Veazey et al., 2003) because this represents an important opportunity to prevent the transmission of HIV in the absence of an effective vaccine and the unreliable use of condoms (Ramessar et al., 2010). Current technology for the manufacture of mAbs using CHO cells may be able to accommodate the demand for HIV-neutralizing mAbs as microbicides in the developed world, but in the long term for sub-Saharan Africa and other underdeveloped countries, the use of plant biotechnology is likely to be a simpler and more robust technology than cell fermentation (Ma et al., 2013).

The human IgG1/x mAb 2G12 has broad neutralizing activity against HIV. The pharmacodynamics of CHO-derived 2G12 have been studied in detail, revealing in vitro activity against a significant proportion of the clinical isolates tested (Binley et al., 2004; Mehandru et al., 2004). The prophylactic potential of 2G12 against viral challenge has been demonstrated in animal models (Baba et al., 2000; Mascola et al., 2000), including efficacy against mucosal infection via the vaginal challenge route. Taking these data into account, it seems likely that the best protection would be achieved by applying the antibody using the same route as virus exposure. The intravaginal application of antibodies aims to provide high concentrations of HIV-neutralizing antibody at the site of infection (Ramessar et al., 2010).

The production of 2G12 has previously been reported in plants although only thus far in the context of proof-of-principle studies that do not address the requirements of process-scale GMP manufacturing. The antibody has been expressed in transgenic maize seeds (Rademacher et al., 2008; Ramessar et al., 2008), Arabidopsis seeds and leaves (Loos et al., 2011), tobacco leaves (Floss et al., 2009; Strasser et al., 2008), and most recently rice seeds (Vamvaka et al., 2015) with similar in vitro biding activity as C2G12 and variable HIV-neutralization efficacy. It has also been produced, along with a panel of other HIV-neutralizing antibodies, by transient expression (Rosenberg et al., 2013; Sainsbury et al., 2010). Although the protein backbone of P2G12 is identical to C2G12 produced in CHO cells, there are differences...
Table 2 Clinical trial schedule of assessments. Each column represents one of seven clinic visits or a telephone communication (TC). Visit no. 3 is the investigational medicinal product (IMP) administration visit, including an overnight stay. For each contact, the assessments are indicated.

<table>
<thead>
<tr>
<th>Visit no</th>
<th>Visit period</th>
<th>1 (Within 2 months before dosing)</th>
<th>2 (Within 2 months before dosing)</th>
<th>3* (IMP administration and immediate follow-up)</th>
<th>4 (Pre-dose)</th>
<th>5</th>
<th>6 (14-16 days)</th>
<th>7 (28-35 days)</th>
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*May occur at any time during the cycle when subject is not menstruating - this is deemed study day 1
in the way plant and animal cells modify glycoproteins (Gomord et al., 2005). From a regulatory perspective, the use of a new manufacturing system also introduces potential differences to the final product, to the extent that P2G12 was regarded as a different product from C2G12 and not a generic or similar product. The specification for P2G12 proposed to the UK MHRA was based on the specification for C2G12, which has the same intended use and which had already been tested in clinical trials both vaginally and intravenously. Additional tests and specifications, for example to determine the nicotine content, were applied to take into account the source of P2G12, and they are consistent with current recognized standards for this stage of product development.

The production of clinical-grade P2G12 in tobacco required the development of an entire production process from first principles, including transformation, the selection of lead events, the establishment of working practices for tobacco cultivation that satisfied the regulatory bodies in Europe, the definition of MSBs and WSBs, the development of a unique GMP-compliant downstream processing infrastructure and finally the completion of a first-in-human clinical trial to test the product for safety. P2G12 is the first GMP-compliant plant-derived mAb to undergo clinical testing in Europe, although a cocktail of three antibodies has previously been demonstrated for C2G12 (West et al., 2009; Qiu et al., 2014). Several nonantibody plant-derived pharmaceuticals have also been produced under GMP conditions for clinical trials (Fischer et al., 2012; Paul et al., 2013) including a recombinant form of the enzyme glucocerebrosidase produced in carrot cells which is currently the only approved product for humans developed by molecular pharming in plants.

A high antibody concentration was selected for clinical testing to overcome the potential dilution and washing effect of vaginal fluid. The maximum solubility of P2G12 in normal saline is 28 mg/mL, and saline was selected as a carrier to avoid the potential complication of mAb capture associated with gel formulations. The purity specification was based on the percentage of monomers and dimers in the API. For a topically applied HIV-neutralizing micobicide, monomers and dimers may both be valuable because dimers have a greater neutralizing potency, as has previously been demonstrated for C2G12 (West et al., 2009; Wu et al., 2013). A 3% limit was placed on larger aggregates or fragments, in line with the C2G12 specification that was used for intravenous administration. Similarly, the biological activity (IC50) specification was based on that previously used for C2G12. The host cell protein specification was placed at the limit of detection for the current detection method using silver-stained SDS-polyacrylamide gels. With further clinical development, a more sensitive assay needs to be developed, but at this stage of product development, the assay described above was considered appropriate.

Protein A, endotoxin and DNA limits were set according to levels that are generally accepted for parenteral products derived from mammalian cells. The nicotine specification was based on recommendations from the European Food Safety Authority (EFSA) on dietary exposure to nicotine (EFSA, 2009). EFSA established an acute reference dose (ARfD) of 0.0008 mg/kg body weight, based on a lowest observed adverse effect level of 0.0035 mg/kg for pharmacological effects after intravenous administration of nicotine. The ARfD equates to 56 μg nicotine for an average adult, and the specification is one order of magnitude (per dose) below this. Because of limited sample availability, formal bioburden analysis of the API was carried out by the IMP manufacturer immediately prior to the sterile filtration step, in addition to the sterility test for the final product specification.

The specification of P2G12 compared favourably with the GMP-compliant manufacturing process developed for C2G12, and matched C2G12 in most parameters, the exception being host cell protein levels, where a more refined assay for plant cell proteins is required to lower the limit of detection.

One result of initial concern was that the HIV-neutralization IC50 for the clinical batch API (batch #090918) was outside specification (21 μg/mL compared to the specification <20 μg/mL). Several potential factors may have contributed to this anomaly. The most important contributor is likely to be the inherent variability of the assay. It is well known that neutralization assays give variable results (Polonis et al., 2008). The syncytium-inhibition assay used here was a cell-based assay in which the presence or absence of multinucleate cells was assessed by microscopy across a series of concentrations of the potential inhibitor. IC50 values are therefore indicative, and a difference of twofold to threefold should not necessarily be regarded as significant. As shown later in the stability studies and in the final analysis of the IMP (all derived from this same API batch), all subsequent samples from this batch were within specification for the HIV-neutralization IC50.

Furthermore, the IC50 value is determined according to the concentration of the inhibitor. As described, a specific assay was developed for P2G12 using surface plasmon resonance spectroscopy to determine specific binding to both protein A and the 2G12 antigen (gp120). However, the C2G12 specification was based on the concentration of C2G12 that was measured differently, namely by absorbance at 280 nm using an extinction coefficient that was specifically calculated for this antibody. When the concentration of P2G12 was determined using the same method, the calculation reduced the concentration of P2G12 in batch #090918 and consequently the IC50 to 19.7 μg/mL, which is within specification. The minor reduction in HIV-neutralization efficacy for the bulk API was considered neither critical nor significant for the objectives of the phase I clinical trial, a conclusion supported by the UK regulatory authority.

An important difference between the specifications for P2G12 and C2G12 was the decision not to include analysis for mycoplasma or viral contamination for P2G12 at this stage of clinical development. The risk associated with potential contaminants such as viruses in plants cultivated in containment is extremely low, and this was therefore reflected in the product specification. Plants do not support the proliferation of animal viruses or mycoplasma, nor do the materials used in the downstream processing strategy that was devised. Therefore, even adventitious contamination is unlikely to lead to a significant increase in virus load. Regarding plant viruses, the original seeds were from a certified source and the plants were constantly monitored for health, but even if such viruses were present, the risks are extremely low because no plant viruses are known to infect or cause adverse reactions in animals despite constant exposure.

The guidelines on Virus Safety Evaluation of Biotechnological IMPs (EMEA/CHMP/BWP/398/498/2005) are applicable to biotechnology-derived IMPs from human or animal cells. Thus, the
specific described process for the manufacture of P2G12 does not technically fall directly under the scope of the guidelines. However, principles from the guidelines were considered for the risk assessment which encompassed the process barriers with respect to their positive contribution to viral safety: raw materials (e.g. soil, fertilizer water), operators (during cultivation, harvesting and processing), process aids (clothes, hygiene and techniques), other materials (chemicals, filters and media), equipment/utilities (heating/ventilation/air conditioning, irrigation and primary processing) and the environment (e.g. the presence of rodents, birds and insects).

Among these risk factors, those with a potential impact on product quality were scrutinized, that is soil, operators, equipment and techniques as well as the potential presence of rodents. In the production process, the soil was a defined and certified material that was stored in closed bags; the other raw materials were all chemically well-defined substances of a specific quality, laid down in certificates of analysis from the vendors; plastic storage bags, filtration and chromatography materials were those commonly used for the manufacture of biologics; all upstream operations were carried out in a closed, access-controlled greenhouse; and downstream processing was carried out in a class C clean room according to Annex 1 of the EU GMP Guide (http://www.emea.europa.eu/ema/). Plant cultivation and management was carried out according to standard operating procedures, and all operators wore adequate protective clothing and were GMP trained. Finally, a nanofiltration step was included in the manufacturing process, which is an established virus removal step that captures even small model paroviruses (Abe et al., 2000).

The clinical trial represented the first ever administration of a plant-derived mAb by the vaginal route in humans and the first use of a GMP-compliant transgenic plant-derived mAb in humans. Therefore, the trial was designed with the primary objective of evaluating the safety and reactogenicity of intravaginally delivered P2G12. For this reason, a simple formulation in normal saline was selected, although any future antibody-based microicide product is likely to comprise a more complex mix of active ingredients and will be formulated differently, thus requiring additional tests.

The main group of subjects received a single application of 28 mg P2G12 in saline. A high mAb concentration was selected to overcome the potential dilution and washing effect of vaginal fluid: each dose was in a volume of approximately 1 mL and 28 mg/mL approaches the maximum solubility of this mAb in saline. This dose in humans was envisaged in the design of the rabbit vaginal irritation study, where a 12.5 mg dose in 0.5 mL was applied. On a body mass basis, this equates to a significant excess in the rabbit study. The average surface area of the rabbit vagina is approximately 15 cm², compared with 90 cm² in humans (Pendergrass et al., 2003). The rabbit dose therefore equated to a human equivalent dose (HED) of 75 mg. In the human study, the starting dose of 7 mg/mL was approximately 9% of the HED, which is in line with typical guidelines for the selection of first-in-human starting doses. The highest dose in humans was more than 2.5-fold lower than the HED.

The clinical study was a placebo-controlled, randomized, double-blind, first-in-human, dose-escalation study to assess the safety of intravaginally-administered P2G12. There were no serious adverse events, nor adverse events related to the IMP in the laboratory tests. No specific anti-P2G12 immunological changes were detected in serum or vaginal fluid following the intravaginal administration of P2G12 at any dose level. The studies separately investigated antibody responses to the P2G12 protein backbone and N-linked glycans. There was some evidence for pre-existing serum IgM and IgA that recognized P2G12. Similar antibody activity was also found against horseradish peroxidase (HRP) and is consistent with the hypothesis that these cross-reactive antibodies are low-affinity antibodies against plant-type N-glycans. Humans are regularly exposed to plant N-glycans both by the mucosal and systemic routes, so this finding is not unexpected.

Serum analysis did not reveal any evidence that P2G12 was systemically absorbed at any of the doses. However, P2G12 was detected in the vaginal cavity for up to 8 h after administration. Because this finding refers to samples that were compromised during transport, 8 h therefore represents the minimum survival time for antibody in the vaginal cavity. Further studies will be required to better characterize the pharmacokinetic properties of P2G12.

Overall, we conclude that P2G12 can be manufactured to a quality equivalent to the C2G12 counterpart produced in CHO cells. The plant-derived antibody was safe and well tolerated in healthy women when administered intravaginally in single doses of up to 28 mg.

This project was established primarily to demonstrate proof-of-concept for the GMP-compliant manufacture of plant-derived clinical-grade mAbs. This was achieved by an academic consortium (Pharma-Planta), supported by public funding, to overcome industry scepticism concerning the likelihood that regulatory compliance could be achieved. Our aim was not to demonstrate the cost efficiencies associated with plant-based production platforms, particularly given the ground-breaking nature of the work. The public funding also allowed the consortium to focus on other important issues related to molecular pharming, including disease targets relevant to the developing world, technology transfer to developing countries, and humanitarian aspects related to new biotechnologies (Ma et al., 2013).

Although all the main goals of the Pharma-Planta project were successfully completed, the results represent just a small step along the long path towards commercialization. It is hoped that by sharing the know-how gathered during the Pharma-Planta project, the development of other molecular pharming products by other parties will be facilitated, thereby accelerating the commercial development of this biotechnology.

**Experimental procedures**

**Development genetics**

**The host plant**

Tobacco is a member of the family Solanaceae and Petit Havana SR1 is a small tobacco variety with day length-independent flower induction. It is substantially homozygous, reflecting many decades of self-pollination. Certified seed was obtained from Lehle Seeds, Round Rock, Texas, USA.

**Transgenes and expression construct**

The genes encoding the light and heavy chains of mAb 2G12 were obtained from Polymun Scientific Immunobiologische Forschung GmbH, Klosterneuburg, Austria. The sequences (including their accompanying signal peptide sequences) were those originally expressed in CHO cells to produce C2G12 previously used in a human phase I trial (Armbruster et al., 2002). A marker gene from reef coral (Discosoma spp.) encoding
a red fluorescent protein (DsRed) (Baird et al., 2000) was fused to the plastid transit signal of the granule-bound starch synthase gene from barley (*Hordeum vulgare*) and placed under the control of the same genetic elements regulating the antibody genes.

The expression vector pTRAp-2G12-Ds was based on the binary plasmid pPAM (GenBank accession number AY027531). The recombinant vector contained the 2G12 heavy and light chain genes and the marker gene DsRed between the left and right borders of the T-DNA, with each gene controlled by the duplicated *Cauliflower mosaic virus* (CaMV) 35S promoter, the *Tobacco etch virus* (TEV) 5′ untranslated region and the CaMV 35S polyadenylation site/terminator (Figure S6). This linkage between the antibody and marker gene was used to identify plant lines with strong antibody expression during the transformation and regeneration procedure. DsRed fluorescence was also used to determine the zygosity of the selected plant line and the stability of transgene expression. The selectable marker gene pat was also included in the T-DNA, allowing the plants to be selected for phosphinothricin resistance, and the four genes were separated by scaffold attachment regions (SARs).

**Generation of the primary transformant**

The tobacco plants were grown in a dedicated, contained production greenhouse at the Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Aachen, Germany. Leaf discs were transformed with A. *tumefaciens* strain GV3101 carrying the helper plasmid pMP90RK (Horsch et al., 1985). Initial transgenic events with high-level DsRed expression were analysed for the presence of 2G12 heavy and light chains by Western blot, using antibodies specific for the human heavy and light chains. Antibody levels in individual primary transgenic T0 lines were quantified by surface plasmon resonance spectroscopy with a BIACORE 2000 instrument (GE Healthcare, Uppsala, Sweden) using protein A coupled to a sensor chip. T0 lines with high antibody levels were self-pollinated to generate subsequent generations of plants.

**Generation of the final transformants**

The antibody level in the T1 generation was monitored in 20–40 individual plants by surface plasmon resonance spectroscopy. In every generation, the plants with the best antibody yields were self-pollinated until the T6 generation. The morphological phenotype, antibody yields and zygosity were determined in each generation, the latter by segregation analysis and the analysis of DsRed fluorescence.

**Transgenic banking system**

The MSB was established and characterized using T5 generation transgenic seed derived from a single transgenic plant line that was selected over four generations. Twenty-five T5 plants from the MSB were self-crossed, and the resulting T6 seed was harvested and divided into 300-mg aliquots. These represented the WSB used to prepare the development batches and the clinical batch of P2G12.

**Genetic characterization and stability**

Genomic DNA was isolated from three individual plants per generation and digested with the restriction enzymes *Nco*I and *Xba*I to excise the complete coding regions of both antibody chains. Southern blots (Worrall, 1998) were used to distinguish between fragments of the anticipated size and truncated copies. The digested DNA (7 μg per lane) was separated by agarose gel electrophoresis and transferred to a nitrocellulose membrane. The immobilized DNA was probed with sequences representing the distal 393 bp of the heavy chain coding region and the distal 313 bp of the light chain coding region, prepared using the AlkPhos Direct Labelling and Detection System with CDP-star (GE Healthcare). Chemiluminescence was detected using an Image-Quant LAS-3000 (GE Healthcare). Plasmid standards containing the transgenes were used as controls to determine the number of transgene copies present in the genome.

Northern blotting was carried out using plants from the T5 to T7 generations to confirm the size of the transcripts produced from the antibody genes. RNA was isolated from three to six individual plants per generation using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), and the assay was carried out as previously described (Maniatis et al., 1982) using the same probes and detection procedure as described above for the Southern blots.

To confirm the identity of the transgenes, the coding regions of the antibody gamma heavy and kappa light chains were sequenced along with small amounts of flanking sequence corresponding to the 5′ and 3′ untranslated regions of the transgenes, thus not extending into the surrounding genomic DNA. For the heavy chain, the flanking sequence extended approximately 180bp upstream and 50bp downstream of the coding region, and for the light chain, the flanking sequence extended approximately 150bp upstream and 50bp downstream of the coding region. This analysis was carried out using three individual plants each from the T5 and T6 generations.

**Manufacturing issues**

Standard operating procedures were prepared for all manufacturing steps (Figure 2), and the process was designed to adopt the principles of good production practice. None of the raw materials used in the manufacturing process were of animal or human origin.

**First production phase**

**Procedures for cultivation.** Transgenic tobacco plants were grown in a dedicated contained production greenhouse on the Fraunhofer IME site, in individual chambers with a controlled environment. The chambers for the production batch were cleaned and disinfected before use and personnel wore protective clothing (laboratory overalls, gloves, hair covers and plastic shoe covers) for all procedures in the greenhouse.

The environmental parameters in the chambers were set to 25/22 °C day/night temperature with a 16-h photoperiod and a minimum of 20 klux in the light cycle using supplementary lighting from high-pressure sodium and metal halide lamps when natural light was insufficient. The relative humidity was maintained at 70%. For each production batch, trays filled with Einheitserde type VM compost (Balster Einheitserdewerk, Fröndenberg, Germany) were seeded with an aliquot of the WSB to a density of 500/m². After 1–2 weeks, a homogeneous population of 1400 plants was selected and transferred into 2.5-L pots containing Einheitserde type T1.5 compost (Balster Einheitserdewerk) at a final density of 8/m². The plants were cultivated on automated Ebb and Flood benches using 0.1% Ferty 2 MEGA (Planta Düngemittel, Regenstauf, Germany) to guarantee a uniform supply of water and nutrients, by periodically pumping a defined nutrient solution from a reservoir into the bench. The plants were inspected daily, and any showing symptoms of unusual growth or disease were removed immediately. No
Harvesting and primary processing: Tobacco leaves were harvested at 6 weeks after sowing. One day in advance of the harvest, approximately 10% of the plants were collected for analysis in terms of biomass, total protein content and antibody yield. The main harvest was carried out manually with visual inspection. Leaves with visible signs of poor health or those in contact with the soil were discarded. The leaves were machine-washed with municipal tap water to remove any particles from the surface and fed into a customized processing device (Sartorius-Stedim Biotech, Göttingen, Germany) which automatically determines the biomass, adds the correct amount of buffer (2.5 volumes of sodium phosphate buffer, pH 7.5) and fills the head space with nitrogen before shredding the leaves, and then continues to rotate the blades slowly after shredding to prevent settling while the crude extract is pumped into the integrated bag-filter/depth-filter section of the device.

The extract was passed through a bag filter to remove large particles, and the filtrate was adjusted automatically to pH 7.5 with NaOH. The partially clarified feed was then passed through a disposable polyethylene/ethylene vinyl alcohol (PE/EVA) bags with NaOH. The partially clarified feed was then passed through a bag-filter/depth-filter section of the device.

Second production phase (downstream processing)

All chemicals used in the manufacturing process were Ph. Eur. (European Pharmacopoeia) grade. The antibody was purified from the clarified extract by protein A chromatography, using two membrane-adsorber modules operating in alternating cycles from the clarified extract by protein A chromatography, using (European Pharmacopoeia) grade. The antibody was purified with 1000 times the 50% tissue culture infectious dose (TCID<sub>50</sub>) of the virus for 1 h at 37 °C before adding the AA-2 cells. Antibody samples were tested in two-fold serial dilutions ranging from 50 to 0.1 μg/mL. Cells were incubated for 5 days before assessment of syncytium formation, and the 50% inhibiting concentration (IC<sub>50</sub>) was then calculated.

Characterization of product and process-related impurities

Residual protein A was quantitated by ELISA using a commercially available kit (Cynogen Technologies, Southport, NC, USA). Host cell (tobacco) proteins were visualized by SDS-PAGE followed by silver staining. DNA contamination was measured using the Threshold<sup>®</sup> DNA assay kit (Molecular Devices, Sunnyvale, CA, USA) by an in-house, GMP-certified contract analytical laboratory (NewLab, Erkrath, Germany). Nicotine levels were determined by gas chromatography mass spectrometry (GC-MS), also by an external, GMP-certified contract analytical laboratory (SGS M-Scan GmbH, Freiburg, Germany). The bioburden of the bulk API was measured in-house, using an assay based on Ph. Eur. recommendations. A 1-ml aliquot was inoculated into tryptic soy broth, and bacterial growth was assessed after incubation at 30 °C for 5 days under aerobic conditions.

Stability testing

Stability testing was carried out on four batches of P2G12, under various standard and accelerated conditions relevant to the proposed clinical use of the product. The frequency of testing was determined by the test intervals recommended by ICH guidelines. Assessments included appearance, proportion of aggregates,
dimers and monomers, antibody concentration and HIV-neutralization activity.

Manufacture of P2G12 as an IMP

The IMP was manufactured by McEwen Laboratories Ltd in a certified facility complying with EU GMP standards for aseptic processing of sterile products. The bulk API was thawed at 5 ± 3 °C for 24–48 h. One sample was used to determine the total viable count (bioburden). The remainder was filtered through two in-line 0.22-μm filters under aseptic conditions, in a Class A positive pressure isolator. Individual aliquots (1.5 mL) were dispensed into presterilized single-dose polypropylene vials (Sarstedt, Nümbrecht, Germany) using a semi-automatic torque head machine which removes the closure, injects the solution using a syringe pump and then recloses the container on a shuttle system within the isolator. The filters were tested for integrity using the bubble point test after use. Single-dose vials were inspected for defects and visible particles, labelled and quarantined at −20 °C pending qualified person (QP) release. The finished IMP was also tested for sterility according to current Ph. Eur. monographs. The analytical tests applied to the API (including antibody content, purity and biological activity assays) were repeated on the IMP.

Stability testing

The suitability of the final sterility filters was assessed by testing the IMP vials and stability after two freeze–thaw cycles. Stability testing was carried out on a bulk API batch that was processed to completion and aliquotted in vials according to the standard operating procedures devised for the IMP. A frozen API batch was thawed at the GMP fill-and-finish facility, aliquotted and then re-frozen at −20 °C. Samples were transported back to Fraunhofer IME and stored at −20 °C for a stability testing regime under standard conditions (according to the criteria described above).

Preclinical toxicity, immunogenicity and pharmacokinetics

One group of ten female rabbits received a single intravaginal administration of P2G12 at a dose of 12.5 mg/animal (total volume 0.58 mL, based on the maximum dose planned in the human clinical trial). A similarly constituted group received a single administration of the same volume of sterile physiological saline. Five animals per group were killed 1 day after administration (day 2), and the remaining five per group were killed on day 8. Body weight, food consumption, ophthalmic characteristics, haematology, blood chemistry, organ weight and macroscopic and microscopic pathology were monitored. Vaginal fluid samples were also taken at necropsy for analysis by ELISA (see details below) to investigate the mucosal immunogenicity and pharmacokinetics of P2G12. Serum samples were also tested for systemic immunogenicity as well as systemic uptake of P2G12 using the same ELISA.

Phase I clinical trial design

The clinical trial was an exploratory phase I, single-centre, double-blind, placebo-controlled randomized, dose-escalation study of a single intravaginal administration of P2G12 at doses of 7–28 mg in a volume of 1 mL. The primary objectives were to evaluate the immediate safety and reactogenicity, and the secondary objectives were to evaluate the pharmacokinetics of P2G12 in blood and vaginal secretions.

The study was sponsored by the University of Surrey and conducted in accordance with good clinical practice (GCP) standards at the Surrey Clinical Research Centre, a phase I-accredited research unit. Eleven healthy female subjects aged 18–50 years, with a body mass index of 18–30 kg/m², were enrolled onto the study. They were required to be in good health as determined by medical history, physical examination, negative serology for HIV, hepatitis and syphilis, negative urethral swab for Neisseria gonorrhoeae and Chlamydia trachomatis and a normal vaginal examination at screening colposcopy with no abnormal cervical smear cytology.

The 11 subjects were randomized into three cohorts by an independent statistician using a SAS-generated allocation list provided to an unblinded pharmacist who assigned the participants. All study staff and participants were blind to the treatment allocations. In the first cohort, two subjects received 7 mg P2G12 dissolved in normal saline and one subject was treated with normal saline only. The subjects were dosed at least 2 h apart. Once the safety data from the 14–16 day postdose follow-up period were reviewed, the second cohort was treated, including two subjects receiving 14 mg P2G12 dissolved in normal saline and one subject treated with normal saline only. Again, the subjects were dosed at least 2 h apart. The third cohort was started following a review of the 14–16 day follow-up safety data, and included four subjects receiving 28 mg P2G12 dissolved in normal saline and one subject treated with normal saline only.

For dosing, subjects were placed in the lithotomy position and 1 mL syringe containing the IMP was inserted fully into the vagina (to hilt at labia) and the IMP completely expelled. Subjects were required to remain recumbent for at least 15 min after dosing before mobilizing.

The entire study lasted 21 weeks (Table 2). Each subject made a total of seven outpatient visits to the clinical site, including one overnight stay after dosing. Following two pretreatment screening visits, IMP administration and immediate follow-up were carried out on days 1 and 2. The subjects attended the clinic on days 3 and 4; there was telephone contact on days 14–16; and then, two final clinic appointments between days 28 and 35.

Adverse events were documented at every visit, coded using the MedDRA dictionary v12.0 by blinded data management personnel and checked by the study physician. Safety assessments included haematology and blood biochemistry panels, urinalysis, vital signs, electrocardiography, vaginal and cervical examinations, and self-reported adverse events. Blood samples and vaginal secretion samples were taken to investigate vaginal and serum P2G12 immunogenicity and P2G12 pharmacokinetics. Blood samples and vaginal secretions were obtained at the following time points: predose, and 1, 4, 8, 24, 48 (±2) and 72 (±2) h postdose. Vaginal secretions were taken using an absorbent Weck-CelTM surgical spear (Medtronic Ltd, Watford, UK) and forceps placed into the vagina for 1 min (Lewis et al., 2011). An additional blood sample was taken at 28–35 days postdose. Blood and vaginal fluid samples were stored at −20 °C before processing.

Anti-P2G12 antibody responses in serum and vaginal fluid

Three ELISA tests were devised to measure specific responses to P2G12. In the first test, the ELISA plates were coated with 2 μg/mL C2G12; in the second, they were coated with 2 μg/mL P2G12; and in the third, they were coated with 2 μg/mL HRP. Only two serum and two vaginal fluid samples were used per patient: the predose and 28–35 day postdose serum samples,
and the predose and 72 h postdose vaginal fluid samples. The P2G12 ELISA was not applied to the vaginal fluid sample because of limited sample volume. Responses were detected with sheep anti-human IgM or IgA HRP-labelled antisera. For the HRP-coated plates, sheep anti-human IgG-labelled antiserum was also used for detection. For the C2G12 and P2G12 ELISAs, the positive control sample was a sheep anti-human IgG antiserum to confirm the presence of 2G12 coating the ELISA plate. A rabbit anti-HRP antiserum was used to demonstrate the presence of plant-glycan structures on P2G12, and the absence of cross-reactive antigen recognition in the case of C2G12. The HRP ELISA was designed to detect antibody responses raised against the plant N-glycans near the hinge region of P2G12. The positive control sample was a rabbit anti-HRP antiserum to confirm HRP coating of the ELISA plate. The negative control was a sheep anti-human IgG antiserum. Anti-rabbit IgG and anti-sheep IgG were also included as negative controls.

P2G12 concentration in serum and vaginal fluid

P2G12 concentration was determined by ELISA, using an anti-2G12 idiotype antibody (Armbruster et al., 2002). Patient serum samples were diluted 1:20. Vaginal fluid was used undiluted. All of the collected samples were assayed. The assay detection limit was 0.06 µg/mL for human serum, and 0.03 µg/mL for human vaginal fluid.

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Author contributions

JM, RF and PC conceived the project and secured the funding. JM and RF jointly coordinated the project and discussions with regulatory bodies. RT was the project manager. JM wrote the manuscript. JD and ML designed and were responsible for the GMP manufacturing process. JM and DL designed the clinical trial; DL and JB were responsible for carrying out the trial. TC was the clinical trials manager and with JM, RF, DL, VI, MP, CvD, PD, PS, RT and BV was responsible for developing and preparing regulatory documentation for the clinical trial application. MS, TR and ES developed and characterized the transgenic plant line. FA performed the glycoanalysis. DK and GS supplied the C2G12 antibody and genes, carried out HIV-neutralization assays and the analysis of preclinical and clinical samples. JM, MP and CvD designed and carried out the analysis of preclinical and clinical serum and vaginal samples.

Conflicts of interest

DK and BV are employees of Polymun Scientific who developed and own the intellectual property for mAb 2G12.

References


**Supporting information**

Additional Supporting information may be found in the online version of this article:

**Figure S1** Southern blot of three individual plants per generation (T5, T6 and T7).

**Figure S2** Northern blot of individual plants from three generations of plants.
Figure S3 Average chemical mass of (a) assembled antibody 2G12 and (b) individual light and heavy chains determined by LC-ESI-MS analysis.

Figure S4 Peptide mapping from on-line LC-ESI-MS analysis of a reduced/carboxymethylated tryptic digest of two antibody batches.

Figure S5 Monosaccharide analysis of P2G12.

Figure S6 Map of the plant expression vector pTRAp-2G12-Ds.