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Characterization of Humanized Antibodies Secreted by *Aspergillus niger*

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Two different humanized immunoglobulin G1(κ) antibodies and an Fab′ fragment were produced by *Aspergillus niger*. The antibodies were secreted into the culture supernatant. Both light and heavy chains were initially synthesized as fusion proteins with native glucoamylase. After antibody assembly, cleavage by *A. niger* KexB protease allowed the release of free antibody. Purification by hydrophobic charge induction chromatography proved effective at removing any antibody to which glucoamylase remained attached. Glycosylation at N297 in the Fc region of the heavy chain was observed, but this site was unoccupied on approximately 50% of the heavy chains. The glycan was of the high-mannose type, with some galactose present, and the size ranged from $Hex_6GlcNAc_2$ to $Hex_{15}GlcNAc_2$. An aglycosyl mutant form of antibody was also produced. No significant difference between the glycosylated antibody produced by *Aspergillus* and that produced by mammalian cell cultures was observed in tests for affinity, avidity, pharmacokinetics, or antibody-dependent cellular cytotoxicity function.

Monoclonal antibodies and derivatives are now in broad use as therapeutic agents, as research tools, as affinity ligands in purification, and in the diagnostics industry. Full-length antibodies are generally manufactured by expression and secretion in mammalian cell cultures. However, therapeutic antibodies are often used in high doses (e.g., in the range of 2 to 15 mg/kg of body weight) to attain activity. Grams of antibody may be required per patient to complete a course of therapy, and hundreds of kilograms of such a large, complex protein must be produced to provide the drug to the patient population. Material needs have driven concerns around manufacturing costs and fermentation capacity. In response, a variety of other production systems, ranging from transgenic plants and animals to microbes, are under evaluation.

Microbial expression systems have been developed successfully for the production of antibody fragments, such as single-chain Fv (ScFv), Fab, and Fab′. For example, quantities of 1 to 2 g of soluble, assembled Fab′ fragments/liter have been demonstrated in the periplasm of *Escherichia coli* (3). Secretion of ScFv fragments into the culture supernatant of *Pichia pastoris* at high titers, e.g., 250 mg/liter and 1.2 g/liter, has been reported by Eldin et al. (6) and by Freyre et al. (8), respectively, and up to 40 mg of secreted Fab/liter has been achieved with this yeast (16, 25). In comparison to antibody fragments, there are very few reports of the production of full-length antibodies in microbial systems and, when reported, the titers have been very low (11, 32). However, Simmons et al. (23) recently reported the periplasmic accumulation of assembled, full-length immunoglobulin G1 (IgG1) in *E. coli* at titers of up to 150 mg/liter.

Several groups previously used filamentous fungi, especially *Trichoderma reesei* and *Aspergillus* species, as hosts for mammalian protein production. We have developed strains of *Aspergillus niger* var. *awamori* which show improved secretion of foreign proteins (30). Typically, the desired mammalian protein is produced as a fusion to the C terminus of a highly secreted native protein, such as *A. niger* glucoamylase or *T. reesei* cellobiohydrolase I, because this strategy can increase titers significantly over those obtained with production as a nonfusion protein (31). A cleavage site (Lys-Arg) recognized by the Golgi apparatus serine proteinase KexB (13) is often used to allow release of the mammalian protein during the secretion process (reviewed in reference 10). This approach has been used by Frenken et al. (7) for the production of an ScFv fragment in *A. niger*. Work with *T. reesei* has demonstrated the production and assembly of an Fab fragment; in this work, the heavy chain (Fd) was expressed as a fusion with native secreted cellobiohydrolase I, while the light chain was not expressed as a fusion protein (19). Titers of up to 150 mg of secreted Fab/liter were observed.

Unlike antibody fragments, full-length IgG1 molecules include an N-linked glycosylation site in the Fc portion of the heavy chain. Production of the antibody in alternative hosts can lead to a lack of glycosylation in bacteria or to altered glycosylation in eukaryotic hosts. It has been shown that the structure of the glycan can be important with respect to pharmacokinetics (33), although nonglycosylated IgG1 has a serum half-life similar to that of wild-type IgG1 (26).

Therapy with a monoclonal antibody may depend on the effector functions that reside in the Fc domain. Recruitment of cytotoxic cells or complement activation may require appro-

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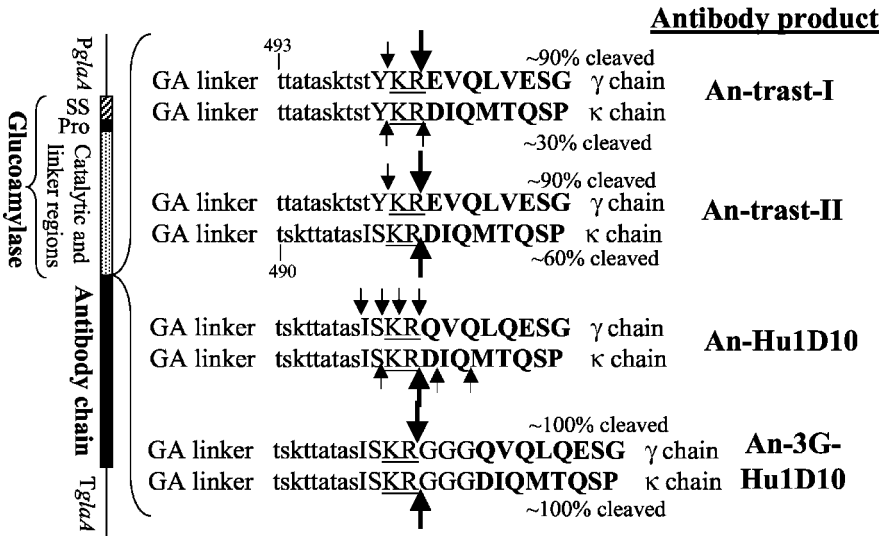

FIG. 1. Schematic representation of the antibody light- and heavy-chain expression cassettes. The vertical bar on the left represents the expression cassette and the encoded proteins. P*glaA*, promoter region of the *glaA* gene; SS, glucoamylase signal sequence; Pro, glucoamylase proregion; T*glaA*, terminator region of the *glaA* gene. To the right are the designations of the various antibody products mentioned in the text. The amino acid sequence at the junction between the C terminus of the glucoamylase (GA) linker region (lowercase) and the N terminus of the antibody heavy (γ) or light (κ) chain (uppercase, bold) is shown for each antibody product. The KexB cleavage site (KR) is underlined. The amino acid numbers for mature glucoamylase are marked on the sequences. For each amino acid sequence, the approximate percentage of that fusion protein that was present in the cleaved form in culture supernatants is shown. For each amino acid sequence, the results of N-terminal sequence analysis of the cleaved form are shown; a large arrow indicates that >50% of the cleaved protein had that N terminus, and a small arrow indicates that <50% of the cleaved protein had that N terminus.

priate glycosylation of the Fc region for optimum interactions with Fcγ receptors or C1q (17; reviewed in reference 14). Alternatively, antigen binding alone may be sufficient for some therapeutic purposes, and effector functions may actually be undesirable. In these situations, a full-length antibody may still be preferred to benefit from the long serum half-life that results from the interaction of the Fc domain with FcRn, the IgG salvage receptor (9). However, glycosylation does not appear to be necessary for antibody interactions with FcRn, and glycan structure may prove similarly irrelevant.

Humanized antibodies consist of the hypervariable complementarity-determining regions derived from rodent antibodies grafted into human framework sequences. In this article, we describe the production of full-length, humanized antibodies in *A. niger* and characterize the products in some detail.

MATERIALS AND METHODS

Expression vector construction. The DNAs encoding the κ- and γ-chain variable regions of trastuzumab were synthesized at Aptagen, Inc. (Herndon, Va.), by using Gene Forge custom gene synthesis technology according to the published amino acid sequence of antibody 4D5-8 (4) and incorporating *Aspergillus* codon bias (GenBank accession numbers AY513485 and AY513484, respectively). The cDNAs encoding human κ- and γ-chain constant regions were amplified by PCR with human leukocyte cDNA (QUICK-Clone cDNA; Clontech Laboratories, Palo Alto, Calif.) as a template. The variable and constant regions were joined and inserted into the expression vectors by standard methods. cDNA clones expressing the entire mature antibody Hu1D10 κ and γ chains (15) were amplified by PCR and inserted into the expression vectors.

Separate expression vectors (available upon request) were constructed for the antibody heavy and light chains. The expression cassette was flanked by the *glaA* (glucoamylase gene) promoter and terminator regions. The open reading frame encoded a fusion protein consisting of the *A. niger* glucoamylase signal sequence, the proregion, most of the mature protein (catalytic domain and part of the linker region but not the C-terminal starch binding domain), a short region including the residues Lys and Arg (cleavage site for *A. niger* KexB), and the mature κ or γ chain of IgG1 (Fig. 1). Either the *A. niger pyrG* gene or the *Neurospora crassa pyr4* gene was used as a selectable marker for *Aspergillus* transformation. Selection was done by complementation of a *pyrG* mutant strain allowing growth on medium lacking uridine (1).

***Aspergillus* strain and transformation.** *A. niger* var. *awamori* strain dgr246ΔGAM:Pyr2$^-$ is a derivative of dgr246 P2 (30) in which the glucoamylase gene has been deleted. The strain thus has deleted *pepA* and *glaA* loci and has been subjected to several rounds of mutagenesis and screening for improved secretion of a mammalian protein (bovine chymosin).

A. niger was transformed by the method of Campbell et al. (2), except that 15 mg of beta-D-glucanase (Interspex Products, San Mateo, Calif.)/ml was used instead of Novozyme 234 to digest mycelial cell walls and release protoplasts.

***Aspergillus* cultures.** For small-scale antibody production, *Aspergillus* was grown in 50-ml starter cultures in 250-ml shake flasks with CSL-fructose medium (containing, per liter, the following components: 100 g of corn steep liquor [50% solids; Roquette America, Inc., Keokuk, Iowa], 1 g of $NaH_2PO_4 \cdot H_2O$, 0.5 g of $MgSO_4$, 100 g of maltose, 10 g of glucose, 50 g of fructose, and 3 ml of Mazu DF60-P [Mazur Chemicals, Gurnee, Ill.] [pH 5.8]). Flasks were incubated at 37°C and 200 rpm for 2 days. Five milliliters of the 2-day-old medium was transferred to 50 ml of Promosoy special medium [containing, per liter, the following components: 70 g of sodium citrate, 15 g of $(NH_4)_2SO_4$, 1 g of $NaH_2PO_4 \cdot H_2O$, 1 g of $MgSO_4$, 1 ml of Tween 80, 2 ml of Mazu DF60-P, 45 g of Promosoy 100 (Central Soya, Fort Wayne, Ind.), and 120 g of maltose]. The pH of the medium was brought to 6.2 with NaOH. The production medium flasks were incubated at 30°C and 200 rpm for 5 days, and supernatant samples were harvested. These culture conditions were used to produce *A. niger*-derived trastuzumab (An-trast-I) for purification.

Fermentation. Selected *Aspergillus* transformants that produced antibody Hu1D10 were grown in 14-liter fermentors with a working volume of 8.0 liters to produce large amounts of material for purification. Each culture was initiated by inoculating 50 ml of CSL-fructose medium with a spore suspension. After 48 to 60 h of incubation at 30°C and 140 rpm, each culture was transferred to 0.8 liters of CSL-fructose medium in a bottom-baffled 2.8-liter Fernbach flask. After overnight incubation at 30°C and 140 rpm, each culture was transferred to 7.2 kg of medium in a 14-liter Biolafitte fermentor. The fermentation medium used for the production of antibody Hu1D10 contained the following components (per liter): $NaNO_3$, 12 g; KCl, 0.5 g; KH_2PO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 2.05 g; $NaH_2PO_4 \cdot H_2O$, 3.5 g; Promosoy 100, 45 g; sodium citrate, 70 g; Tween 80, 1 g; Mazu DF204, 0.5 g; NETO 7350 (a high-maltose corn syrup; A. E. Staley Manufac-

turing, Decatur, Ill.), 41 g; and trace elements solution, 1 ml. The pH was maintained at 5.5 by the addition of 28% (wt/wt) NH_4OH. The fermentation medium used for the production of antibody 3G-Hu1D10 contained the following components (per liter): $(NH_4)_2SO_4$, 15 g; $MgSO_4 \cdot 7H_2O$, 1 g; $NaH_2PO_4 \cdot H_2O$, 1 g; Promosoy 100, 45 g; sodium citrate, 70 g; Tween 80, 1 g; Mazu DF204, 0.5 g; and maltose, 120 g. The initial pH of 6.2 was allowed to fall to 5 and then was maintained by the addition of 28% (wt/wt) NH_4OH. The fermentors were run at 30°C with an overpressure of 0.5 bar (1 bar = 10^5 Pa) and an airflow of 5 standard liters per minute. Agitation was ramped from 250 to 750 rpm to minimize splashing. Glucose at 60% (wt/wt) was fed to maintain an excess concentration in the fermentors, as monitored by using a glucose oxidase assay kit (Instrumentation Laboratory Co., Lexington, Mass.). Expression from the *glaA* promoter was induced under these conditions even though glucose, rather than maltose, was fed.

Antibody detection. Samples of culture supernatants were mixed with an appropriate volume of 2× sample loading buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with precast gels according to the manufacturer's instructions (NuPAGE bis-Tris electrophoresis system; Invitrogen Corporation, Carlsbad, Calif.). Either the gels were stained for protein with Coomassie brilliant blue stain or the protein was transferred to membrane filters by Western blotting (28). Human κ light chains and γ heavy chains were visualized on Western blots by sequential treatment with goat anti-human κ light-chain (bound and free) antibody or goat anti-human IgG Fc followed by rabbit anti-goat IgG conjugated with horseradish peroxidase (HRP). HRP color development was brought about by incubation with H_2O_2 and 4-chloro-1-naphthol.

Quantification of antibody in culture supernatants was performed by enzyme-linked immunosorbent assays (ELISAs) with quantification kits from Bethyl Laboratories, Inc. (Montgomery, Tex.). For light-chain detection, goat anti-human κ (bound and free) was used to coat microtiter plate wells and, after sample addition and washing, the bound light chain was detected with goat anti-human κ (bound and free)– HRP conjugate followed by the color development reaction. For heavy-chain detection, goat anti-human IgG Fc was used to coat microtiter plate wells, and goat anti-human IgG Fc– HRP conjugate was subsequently added. For the detection of assembled antibody or the Fab′ fragment, we used goat anti-human κ (bound and free) followed by goat anti-human IgG Fc–HRP conjugate or goat anti-human IgG (Fab specific)– HRP conjugate, respectively. Serial dilutions of known concentrations of human κ and γ chains were used as standards for quantification purposes.

Antibody purification. Antibody was purified from shake flask culture supernatants after the removal of particulates by centrifugation and buffer exchange into phosphate-buffered saline on 10-ml Econo-Pac 10 DG desalting columns (Bio-Rad Laboratories, Hercules, Calif.). Affinity chromatography on a HiTrap protein A HP chromatography column (Amersham Pharmacia, Piscataway, N.J.) then was performed by following the manufacturer's protocol.

Antibody was purified from fermentor cultures as follows. Fungal cells were removed from culture broth by filtration through a cellulose pad (K900; Seitz) with FW12 diatomaceous earth (Eagle Pitcher) as a filter aid. The filtered broth was concentrated approximately sevenfold by tangential ultrafiltration with a cellulose membrane with a 30,000-molecular-weight cutoff (Prep/Scale TFF; Millipore). To remove particulates, the concentrate was centrifuged at 25,000 × *g* for 15 min, and the supernatant was filtered through a series of membranes; each membrane had a smaller pore size than the previous membrane, ending with a 0.2-μm-pore size. IgG1 was purified from the supernatant by hydrophobic charge induction chromatography (HCIC) performed with the aid of a high-performance liquid chromatography (HPLC) system (AKTA Explorer 10; Amersham Biosciences, Uppsala, Sweden). HCIC provided the ability to separate antibody molecules from other supernatant proteins and from glucoamylase fusion proteins.

HCIC was carried out on a column containing MEP HyperCel (Ciphergen Biosystems) medium. The column was equilibrated with 50 mM Tris– 200 mM NaCl (pH 8.2) buffer. A supernatant, adjusted to pH 8.2, was applied to the column at a linear flow rate of 100 cm/h. After the column was washed with five column volumes of equilibration buffer, bound molecules were eluted by incrementally decreasing the pH. Two column volumes of each of the following buffers were delivered to the column at 200 cm/h, in the order listed: 100 mM sodium acetate (pH 5.6), 100 mM sodium acetate (pH 4.75), 100 mM sodium acetate (pH 4.0), and 100 mM sodium citrate (pH 2.5). Free IgG1 eluted within the pH range of 4.5 to 5.5 and was immediately neutralized with 1 M Tris (pH 8.2) buffer. The purity of the antibody exiting the column was assessed by SDS-PAGE.

Size exclusion chromatography (SEC) was subsequently performed by using an AKTA Explorer system with a HiLoad 26/60 column and Superdex 200 preparation-grade medium (Amersham Biosciences). The flow rate was maintained at 17 cm/h. After the column was equilibrated with 20 mM sodium acetate–136 mM NaCl (pH 5.5), a 6.5-ml sample was driven through the column with one column volume of equilibration buffer.

Amino-terminal sequences. Following SDS-PAGE, proteins was transferred to polyvinylidene difluoride membranes, and amino-terminal sequence analysis by Edman degradation was performed at Argo BioAnalytica, Inc.

Monosaccharide analysis. The monosaccharide composition of the antibody sample was determined at Charles River Laboratories (Worcester, Mass.) by HPLC analysis of sugars released by acid hydrolysis.

Proteolytic digestion and LC-MS analysis. Peptide mapping and glycan analysis by reverse-phase HPLC–mass spectrometry (MS) were performed at Charles River Laboratories. Antibody was reduced and carboxymethylated by diluting 1 mg in 1 ml of 6 M guanidine HCl– 20 mM Tris base– 2 mM EDTA (pH 8.3). After the addition of 20 mmol of dithiothreitol and incubation for 2 h at 37°C, 40 mmol of iodoacetamide was added and incubation was continued for a further 2 h at 37°C in the dark. The antibody was dialyzed overnight in 50 mM ammonium bicarbonate– 1 M urea (pH 8.3). The sample then was removed from the dialysis cassette, trypsin was added to provide an enzyme/antibody ratio of 1:25 (wt/wt), and the sample was incubated at 37°C for 24 h. An HP1100 HPLC system coupled to a Q-TOF II electrospray ionization mass spectrometer (Micromass Inc., Elstree, United Kingdom) was used for liquid chromatography (LC)-MS and LC-MS– MS analyses. MS data were collected from 200 to 2,500 *m/z*. For individual peak MS-MS analysis, data were collected from 50 to 2,000 *m/z*. For reverse-phase HPLC, a Vydac C_{18} column was used at 35°C, and the flow rate was 0.2 ml/min with 0.01% trifluoroacetic acid in deionized water as mobile phase A and 0.007% trifluoroacetic acid in acetonitrile as mobile phase B. Peptide mapping analysis was performed by using the Micromass ProteinLynx 3.5 program.

Cell proliferation assay. The ability of trastuzumab produced by *Aspergillus* to inhibit the proliferation of human breast adenocarcinoma cell line SK-BR-3 (ATCC HTB-30), which overexpresses HER2, was compared to that of commercially available trastuzumab (Herceptin; Genentech, South San Francisco, Calif.) by using assays similar to those described by Hudziak et al. (12) and Carter et al. (4). Human epidermoid carcinoma cell line A-431 (ATCC CRL-1555), which expresses low levels of HER2 and high levels of the epidermal growth factor receptor, was used as a control (see reference 12 and references therein). SK-BR-3 cells were maintained in McCoy's 5a medium containing 15% fetal bovine serum (FBS) and A-431 cells were maintained in Dulbecco minimal essential medium containing 10% FBS (all media from Invitrogen) in a 5% CO_2 incubator. Cells were detached with trypsin and suspended in complete medium at a density of 1.8×10^4/ml. Aliquots of 100 μl, 1.8×10^3 cells, were placed in 96-well microtiter plates, and the cells were allowed to adhere for 6 h. Aliquots of 100 μl of medium containing twofold serial dilutions of antibody from 20 to 0.078 μg/ml (final concentrations of 10 to 0.039 μg/ml in the miocrotiter plates) or medium alone then were added to the wells. After 72 h, relative proliferation was measured by using a CellTiter 96 aqueous one-solution cell proliferation assay (Promega Corporation, Madison, Wis.) according to the manufacturer's instructions. All assays were performed in triplicate.

Flow cytometry. The human Burkitt's lymphoma-derived cell line Raji (ATCC, Manassas, Va.), which expresses the HLA-DR β-chain allotype recognized by antibody Hu1D10 (15), was maintained in RPMI 1640 (Gibco BRL, Grand Island, N.Y.) containing 10% FBS (HyClone, Logan, Utah) in a 7.5% CO_2 incubator. For the competition experiments, a mixture of fluorescein isothiocyanate (FITC)-labeled antibody NS0-Hu1D10 (0.25 μg/test) and competitor antibody (serial twofold dilutions of NS0-Hu1D10 [Hu1D10 derived from a mouse myeloma NS0 cell culture] or *Aspergillus*-derived Hu1D10 starting at 6.25 μg/test) in fluorescence-activated cell sorting staining buffer (phosphate-buffered saline containing 1% bovine serum albumin [BSA] and 0.2% sodium azide) was added to Raji cells (5×10^5 cells/test) in a final volume of 100 μl. After incubation on ice for 30 min, the cells were washed three times with fluorescence-activated cell sorting staining buffer and analyzed by flow cytometry with a FACScan (Becton Dickinson, San Jose, Calif.). The competitor concentration (nanograms per test) was plotted against the mean channel fluorescence.

Apoptosis assay. To measure the abilities of antibody NS0-Hu1D10 and the *Aspergillus*-derived Hu1D10 antibodies to induce apoptosis, Raji cells resuspended at 5×10^5 cells/ml in RPMI 1640 containing 10% FBS were incubated with 2 μg of antibody at 37°C for 5 or 24 h in quadruplicate. The cells then were washed three times with the binding buffer provided in an apoptosis detection kit (Pharmingen, San Diego, Calif.) and were stained with FITC-conjugated annexin V and propidium iodide according to the manufacturer's protocol. Cell death was determined by two-color flow cytometry. Percent apoptosis was defined as the sum of the percentage of cells stained with annexin V alone (apoptotic cells)

and the percentage of cells stained with both annexin V and propidium iodide (necrotic cells). Relative cell fluorescence was analyzed with a FACScan.

ADCC. Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by using Ficoll-Paque PLUS lymphocyte isolation solution (Amersham Biosciences). Antibody-dependent cellular cytotoxicity (ADCC) with antibody Hu1D10 was analyzed by using a lactate dehydrogenase (LDH) detection kit (Roche Molecular Biochemicals, Indianapolis, Ind.) with PBMCs as effector cells and Raji cells as target cells. Target cells were washed and resuspended in RPMI 1640 containing 1% BSA and added to 96-well U-bottom plates with the desired concentrations of antibody NS0-Hu1D10 and the *Aspergillus*-derived Hu1D10 antibodies in 100-μl volumes per well. The plates were incubated for 30 min at 37°C for opsonization. Effector cells in 100 μl of RPMI 1640 containing 1% BSA then were added to each well at an effector/target ratio of 40:l. After 4 h of incubation at 37°C, all plates were centrifuged, and cell-free supernatants were incubated with the LDH reaction mixture in separate 96-well flat-bottom plates for 30 min at 25°C. The absorbance of reaction samples was measured at 490 nm. Antibody-independent cellular cytotoxicity was measured by adding effector and target cells in the absence of antibodies. Spontaneous release was measured by adding only target or effector cells. LDH release was negligible when Raji cells were incubated with antibody Hu1D10 in the absence of effector cells. Maximal release was measured by adding 2% Triton X-100 to target cells. Percent lysis was determined with the following equation: [(LDH release of sample − spontaneous release of effector cells − spontaneous release of target cells)/(maximal release of target cells − spontaneous release of target cells)] × 100. Each condition was examined in duplicate.

Pharmacokinetics. Two groups of Sprague-Dawley rats (weight range, approximately 250 to 300 g) received, per kilogram of body weight, an intravenous bolus dose of 2 mg of *A. niger*-derived trastuzumab (three rats) or commercially available trastuzumab (four rats). Animals were dosed according to individual body weight with an antibody preparation that had been diluted to a final concentration of 0.9 mg/ml. Blood samples to provide serum (0.5 ml/sample) were collected at 0, 1, 4, 8, 24, 48, 72, and 96 h and at 7, 12, and 14 days postdose. Serum was prepared by centrifugation of the blood samples within 30 min of collection. The serum was decanted, and the serum samples were stored at −80°C. Human IgG1 levels in these serum samples were measured by ELISAs as described above. The minimum dilution of the serum samples in these assays was 1:800, and the limit of detection was 2.5 ng/ml for commercially available trastuzumab added to serum from a nondosed rat.

RESULTS

***Aspergillus* transformation.** Separate *Aspergillus* expression vectors were constructed for the antibody κ light chains and γ1 heavy chains of trastuzumab or Hu1D10. The expression cassettes consisted of the *A. niger glaA* promoter and an open reading frame that encoded a fusion protein consisting of the signal sequence and 6-amino-acid proregion of *A. niger* glucoamylase, the mature glucoamylase protein up to the linker region (53 kDa, not including glycosylation), a cleavage site for KexB, and the mature antibody light or heavy chain (Fig. 1). The light-chain and heavy-chain expression vectors, one of which included the *A. niger pyrG* gene or the *N. crassa pyr4* gene as a selectable marker, were used to generate cotransformants of strain dgr246ΔGAM:Pyr2$^-$ of *A. niger* var. *awamori*. The exact amino acid sequence present at the junction between glucoamylase and the immunoglobulin chain is shown in Fig. 1 for four different transformants. The antibodies produced by these four different transformants were designated An-trast-I, An-trast-II, An-Hu1D10, and An-3G-Hu1D10.

Antibody in shake flask culture supernatants was detected by visualization of protein bands by SDS-PAGE or by ELISAs. In this way, individual transformants producing high levels of antibody and balanced amounts of light and heavy chains could be identified. For An-trast-I, 41 transformants were screened in this manner after cotransformation with the glucoamylase–light-chain vector bearing the *N. crassa pyr4* gene and the glucoamylase– heavy-chain vector with no selectable marker. For An-trast-II, 36 transformants were screened after cotransformation with the glucoamylase– light-chain vector bearing the *A. niger pyrG* gene and the glucoamylase– heavy-chain vector with no selectable marker. For An-Hu1D10, 36 transformants were screened after cotransformation with the glucoamylase– light-chain vector bearing the *A. niger pyrG* gene and the glucoamylase– heavy-chain vector with no selectable marker. For An-3G-Hu1D10, 21 transformants were screened after cotransformation with the glucoamylase– light-chain vector with no selectable marker and the glucoamylase– heavy-chain vector bearing the *A. niger pyrG* gene as a selectable marker. The transformant with the highest titer of trastuzumab produced up to 0.9 g of IgG1(κ)/liter in 50-ml shake flask cultures. In contrast, the transformants with the highest titer of Hu1D10 produced approximately 0.2 g/liter. The vectors were shown by Southern analysis to integrate at one or more random sites in the genome and in multiple (approximately three to five) copies (results not shown). Due to these differences in copy number and integration site, it is not possible to directly compare titer data between different transformants.

It was also possible to transform only the light- or heavy-chain vector into *Aspergillus*. Up to 1.5 g of secreted trastuzumab light chain/liter or 0.33 g of trastuzumab heavy chain/liter was detected by ELISAs for transformants expressing only one or the other of the individual antibody chains. SDS-PAGE analysis showed that the light chain was predominantly intact but that the heavy chain was prone to degradation.

An Fab′ form of trastuzumab was produced by coexpression of the light chain and a truncated form of the heavy chain. The same glucoamylase– light-chain expression vector as that described above was used for this purpose. In the glucoamylase–heavy-chain expression vector, the coding region for the heavy chain terminated after the codon for Cys229 (the second Cys residue in the antibody hinge region between C_H1 and C_H2) with the addition of two Ala codons and a stop codon. The highest titers of the trastuzumab Fab′ form measured in shake flask cultures by ELISAs were approximately 1.2 g/liter.

Antibody assembly and cleavage. Nonreducing SDS-PAGE revealed a series of bands in supernatant samples of transformed strains without purification; these bands were absent from parental strains (Fig. 2A). The antibody was purified from supernatants by protein A affinity chromatography, and this purified material was analyzed by SDS-PAGE under reducing conditions as shown in Fig. 2B. The identity of the bands was confirmed by Western blot and MS analyses (results not shown).

Assembled antibody of 150 kDa was observed on nonreducing SDS-PAGE for all transformants. For An-3G-Hu1D10 (Fig. 2A, lane 3), this was the only major band visible on the gel, other than the glucoamylase released from the antibody fusion proteins at approximately 60 kDa and the native secreted proteins that were also produced by the parental strain (Fig. 2A, lane 4). Thus, cleavage of glucoamylase from the antibody chains appeared to be complete. In addition to the 150-kDa form, a series of protein bands (at approximately 200, 250, 300, and 350 kDa), interpreted as assembled antibody with from one to four glucoamylase molecules attached to the N termini of the individual antibody chains, were observed for An-trast-I (data not shown) and for An-trast-II and An-Hu1D10 (Fig. 2A). For these antibodies, it was evident that

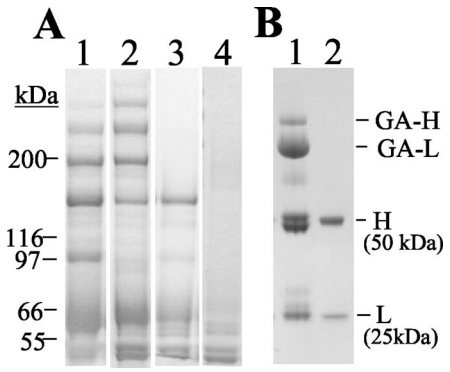

FIG. 2. SDS-PAGE of secreted proteins stained with Coomassie brilliant blue. (A) Gel run under nonreducing conditions; each lane contained 10 μl of *Aspergillus* culture supernatant. Lane 1, An-trast-II; lane 2, An-Hu1D10; lane 3, An-3G-Hu1D10; lane 4, untransformed parent strain. (B) Antibody purified by protein A affinity chromatography. The gel was run under reducing conditions. Lane 1, An-trast-I; lane 2, commercially available trastuzumab. GA-H, glucoamylase–heavy-chain fusion protein; GA-L, glucoamylase– light-chain fusion protein; H, antibody heavy chain; L, antibody light chain.

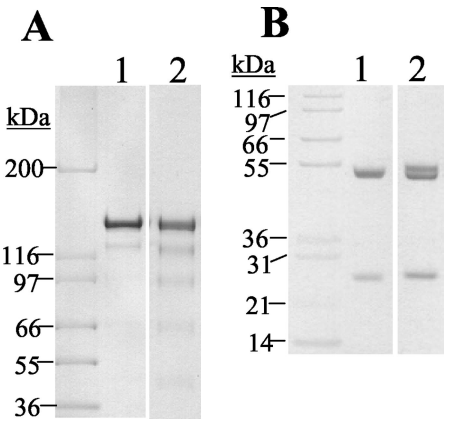

FIG. 3. SDS-PAGE of purified An-trast-I. (A) Gel run under nonreducing conditions. Lane 1, commercially available trastuzumab; lane 2, An-trast-I. (B) Gel run under reducing conditions. Lane 1, commercially available trastuzumab; lane 2, An-trast-I.

cleavage of the glucoamylase-antibody fusion proteins was not complete. This finding was also apparent on reducing SDS-PAGE following protein A chromatography; glucoamylase–light-chain and glucoamylase– heavy-chain fusion proteins were observed in addition to free light and heavy chains (exemplified by An-trast-I in Fig. 2B). Glucoamylase– light-chain fusion cleavage was apparently less efficient than glucoamylase– heavy-chain fusion cleavage. Clearly, antibody assembly and disulfide formation occurred even though glucoamylase was attached to the N termini of the four chains of an IgG1 molecule.

Two forms of the antibody heavy chain released from glucoamylase were observed on reducing SDS-PAGE for all transformants (Fig. 2B). One form was determined to be glycosylated because endoglycosidase H treatment caused a loss of the lower-mobility heavy-chain band. The other form of the heavy chain was shown to be nonglycosylated by MS analysis, since the mass of the relevant tryptic peptide was consistent with the presence of unmodified Asn297 (see below).

Aglycosylated antibody was produced by coexpression of the trastuzumab light chain and a mutant form of the heavy chain in which the codon for Asn297 was changed to encode a glutamine residue. In this instance, only one form of the heavy chain was seen on reducing SDS-PAGE, and this band had the same mobility as the higher-mobility (nonglycosylated) band described above (results not shown). Approximately 0.1 g of aglycosylated trastuzumab/liter was produced.

N-terminal sequence analysis by Edman degradation of the antibody chains released from glucoamylase was performed after purification as described below. The sequences around the KexB cleavage site, the position of actual cleavage, and the approximate frequency of cleavage are shown in Fig. 1. It was observed that the amino acids immediately next to the amino-terminal side of the KexB site could influence cleavage efficiency, as demonstrated previously by Spencer et al. (24). For example, inclusion of the sequence Ile-Ser before the KexB cleavage site in An-trast-II improved the percentage of light chain that was separated from glucoamylase from approximately 30% to approximately 60%, as judged by the intensity of bands on SDS-PAGE under reducing conditions. In one instance, antibody Hu1D10 was expressed with three glycine residues included immediately after the Lys and Arg residues of the KexB cleavage site to produce An-3G-Hu1D10. Analysis by SDS-PAGE showed that efficient cleavage had occurred in this instance, and a single, 150-kDa band representing assembled antibody was observed under nonreducing conditions (Fig. 2A). N-terminal sequence analysis also showed that the fidelity of cleavage at the desired site immediately after the Lys and Arg residues was improved in this instance. Similar heterogeneity of the cleavage site and improvement by insertion of three glycine residues at the C-terminal side of the cleavage site have been reported elsewhere (24).

Purification of free antibody. As mentioned above, protein A affinity chromatography served to capture antibody from *A. niger* culture supernatants. Both free antibody and any antibody to which glucoamylase remained attached were copurified by this method. However, a method that could separate free antibody from glucoamylase-antibody fusion proteins was also sought. HCIC with MEP HyperCel was previously investigated as an alternative to protein A affinity chromatography for antibody capture and purification (20). We adapted this procedure as described in Materials and Methods and found that after loading of the column at pH 8, free antibody could be eluted within the pH range of 4.5 to 5.5, whereas glucoamylase-antibody fusion proteins did not elute until the pH was lowered further.

Fractions from HCIC containing free An-trast-I antibody (that had been produced in shake flask cultures) were pooled and further purified by protein A affinity chromatography followed by SEC to remove degraded or incompletely assembled antibody. Alternatively, a two-step purification procedure with HCIC and SEC but without the protein A affinity chromatography step was used to purify An-Hu1D10 and An-3G-Hu1D10 (that had been produced in fermentors). Antibody preparations were generated by these methods for further characterization, and an example of purified An-trast-I analyzed by SDS-PAGE is shown in Fig. 3. Under nonreducing

conditions, a single prominent antibody band at 150 kDa was observed along with some minor bands of lower molecular masses, which may include antibodies lacking one light chain (125 kDa) and a heavy-chain dimer (100 kDa). Under reducing conditions, only the light chain and the heavy-chain doublet (glycosylated and nonglycosylated forms) were observed. Glucoamylase– light-chain and glucoamylase– heavy-chain fusion proteins were not detected.

For Fab′ production, the antibody fragment released from glucoamylase could be purified by the same HCIC procedure. In this instance, subsequent SEC separated two protein fractions, one with an apparent molecular mass on nonreducing SDS-PAGE of approximately 50 kDa and one of 100 kDa (results not shown). On reducing SDS-PAGE, these two fractions were indistinguishable, both showing a single band of comigrating heavy chains (Fd′) and light chains of 25 kDa. Thus, these fractions presumably represented Fab′ (approximately 80% of the total) and $F(ab')_2$ (20% of the total).

Antigen binding. The binding of An-trast-I to the extracellular domain of the HER2 (ErbB2) receptor was tested by measuring the dose-dependent effects on the proliferation of SK-BR-3 breast cancer cells, which express high levels of HER2 (12, 4). No obvious difference was observed between the antiproliferative effects of An-trast-I and those of commercially available, Chinese hamster ovary (CHO) cell-derived trastuzumab. For both antibodies, the maximal reduction of proliferation (to almost 50% the proliferation of SK-BR-3 cells without the addition of antibody) was reached at an antibody concentration of 0.625 μg/ml.

A competitive binding assay was performed to examine the affinity of the two types of *Aspergillus*-derived Hu1D10 antibodies (An-Hu1D10 and An-3G-Hu1D10) relative to that of control antibody NS0-Hu1D10 for the HLA-DR β chain on the surface of Raji cells. The cells were incubated with a fixed amount of FITC-conjugated NS0-Hu1D10 and various amounts of unlabeled NS0-Hu1D10 or either of the two *Aspergillus*-derived Hu1D10 antibodies. The relative cell fluorescence was measured by flow cytometry. No clear difference in binding to Raji cells was observed among NS0-Hu1D10 and the *Aspergillus*-derived Hu1D10 antibodies (Fig. 4), indicating that the production of Hu1D10 in *A. niger* had little effect on the structure of its antigen binding site.

It has been shown that Hu1D10 produced in NS0 cells can induce the apoptosis of Raji cells through cross-linking of HLA-DR β chains on the cell surface (15). The avidities of the Hu1D10 antibodies were compared by monitoring the level of apoptosis induced by different antibody samples in a population of Raji cells. Apoptosis was monitored by staining with FITC-annexin V and propidium iodide after incubation with antibody NS0-Hu1D10 or either of the two *Aspergillus*-derived Hu1D10 antibodies for 5 and 24 h (Fig. 5). There was very little difference in the abilities of antibody NS0-Hu1D10 and the *Aspergillus*-derived Hu1D10 antibodies to induce apoptosis in Raji cells.

Peptide mapping and glycan analysis. Monosaccharide analysis was performed to determine the identities of sugars associated with An-trast-I. The numbers of monosaccharide molecules observed per IgG1 molecule were 1.5 *N*-acetylglucosamine (GlcNAc), 9 mannose (Man), and 2.6 galactose. These were the average values from two determinations. Glycans of filamentous fungi are known to be of the high-mannose type, and terminal galactose, in the furanose form (Galf), has been observed on N-linked glycans from *A. niger* (29).

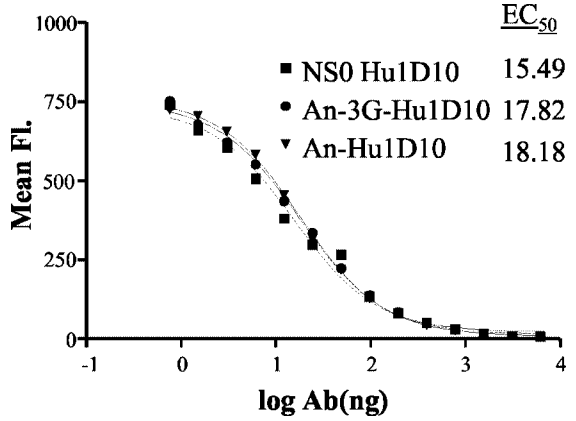

FIG. 4. Competition binding assay. The amount of antibody (Ab) NS0-Hu1D10, An-3G-Hu1D10, or An-Hu1D10 that was mixed with 250 ng of FITC-labeled NS0-Hu1D10 is shown on the *x* axis. After incubation of the antibody mixtures with Raji cells, the resulting mean fluorescence (Fl) of the cell population was measured by flow cytometry and is shown on the *y* axis. EC_{50}, 50% effective concentration.

An-trast-I was digested with trypsin, and the peptide fragments were separated by reverse-phase HPLC and analyzed by MS. Tryptic fragments that represented 99% of the antibody molecule were identified on the basis of mass by LC-MS analysis, and the amino acid sequences determined for these fragments by MS-MS analysis represented 77% of the antibody. The peptide from the heavy chain that includes residue N297, the single potential site of N-linked glycosylation, was identified. As mentioned above, an unmodified Asn residue was present in a subpopulation of this peptide. In the other subpopulation, the peptide was modified in a manner consistent with the addition of a glycan. LC-MS and MS-MS data showed that the glycan structure appeared to be of the high-mannose type, with the number of hexose residues per glycan ranging from 6 to 15 and the most abundant containing 9 hexose residues (that is, $Hex_9GlcNAc_2$). The data also indicated the presence of peptides corresponding to both the intact C ter-

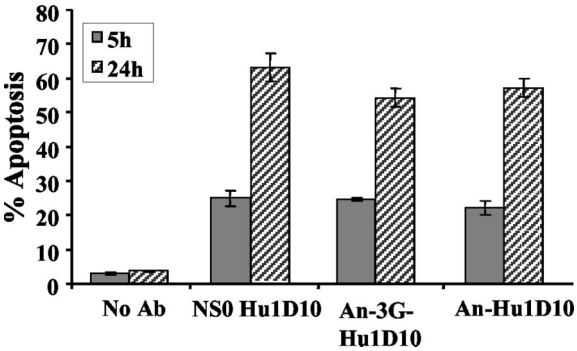

FIG. 5. Apoptosis assay. The percentages of Raji cell populations that underwent apoptosis after incubation for 5 or 24 h with NS0-Hu1D10, An-3G-Hu1D10, or An-Hu1D10 are shown. The data are averages from quadruplicate experiments, and the error bars indicate standard deviations.

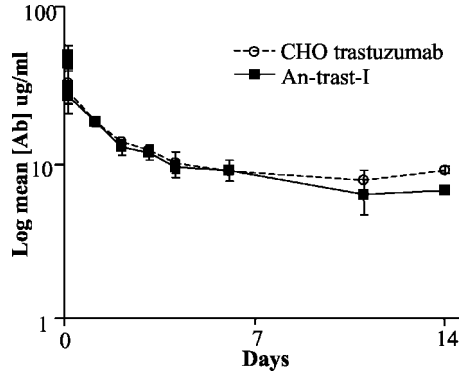

FIG. 6. Pharmacokinetics in rats. The mean concentrations of human IgG1(κ) antibody (Ab) in serum samples at various time points after injection of rats with either CHO cell-derived trastuzumab (four rats) or An-trast-I (three rats) are shown. Standard deviations are indicated by error bars.

minus of the heavy chain and a form lacking the terminal Lys residue. This modification is seen in IgG1 preparations obtained from mammalian cell cultures. No evidence for glycosylation at sites other than Asn297 was observed, but we cannot exclude the possibility that a subpopulation of antibody molecules had additional glycan.

In combination, the information from monosaccharide analysis and data from MS analysis suggested that the most abundant glycan structure was $Galf_2Man_7GlcNAc_2$. However, both simpler and more complex structures were also present.

Pharmacokinetics. Two groups of rats received 2 mg of antibody/kg as a single intravenous bolus dose; one group (three rats) was dosed with An-trast-I, and one group (four rats) was dosed with CHO cell-derived trastuzumab. Serum samples were collected at various time points over 14 days. The concentration of human IgG1(κ) in serum was measured by ELISAs. Similar serum concentration-versus-time profiles were observed for An-trast-I and trastuzumab (Fig. 6). A noncompartmental analysis of the data was performed. Given the half-life of trastuzumab of several weeks, the present study was too short to obtain an accurate estimate of this parameter. However, the parameters commonly used to evaluate bioequivalence, namely, the mean peak concentration of antibody in serum (C_{max}) and the area under the concentration-time curve up to the last measured concentration (AUC_{last}), were comparable for the two antibodies. Specifically, the C_{max} values (mean and standard deviation) were 49.5 ± 6.2 μg/ml for the CHO cell-derived antibody and 48.3 ± 3 μg/ml for An-trast-I, and the AUC_{last} values were 153 ± 4.4 μg · h/ml for the CHO cell-derived antibody and 143 ± 11 μg · h/ml for An-trast-I. These results indicated that the fungal expression of trastuzumab did not significantly affect the pharmacokinetic disposition of the antibody in vivo.

ADCC. NS0-Hu1D10 can mediate the lysis of Raji cells through ADCC (15). It has been shown that the removal of carbohydrates attached at position 297 in the heavy chain abolishes the ADCC activity of human IgG1 antibodies (5). In addition, recent studies have shown that the removal of fucose in attached carbohydrates results in an increase in the ADCC activity of human IgG1 antibodies (21, 22). Since the carbohydrate structure of *Aspergillus*-derived antibodies is different from that of mammalian cell-derived antibodies, the abilities of the two *Aspergillus*-derived Hu1D10 antibodies and antibody NS0-Hu1D10 to induce ADCC were examined by using Raji cells as targets and PBMCs as effectors.

Human PBMCs from two different donors were used in the analysis. With donor A (Fig. 7A), the maximal cytotoxicity level reached nearly 40% with any of the three Hu1D10 antibodies. In this particular experiment, An-Hu1D10 induced cytotoxicity slightly better than the other two Hu1D10 antibodies. Between NS0-Hu1D10 and An-3G-Hu1D10, however, there was no clear difference in the induction of cytotoxicity. With donor B (Fig. 7B), the maximal cytotoxicity level was

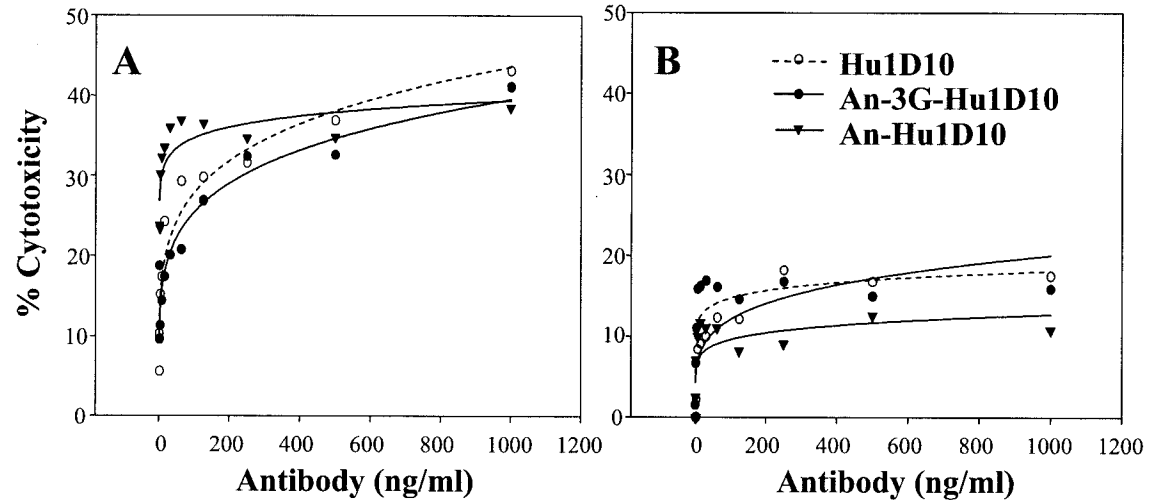

FIG. 7. ADCC assay. Raji cells and human PBMCs were mixed at a ratio of 1:40 and incubated with the amounts of NS0-Hu1D10, An-3G-Hu1D10, or An-Hu1D10 indicated on the *x* axes. The percentages of Raji cell populations that exhibited ADCC are shown on the *y* axes. (A) PBMCs obtained from one human donor. (B) PBMCs obtained from a second human donor. The data are averages from duplicate experiments.

between 15 and 20%, and the differences among the three Hu1D10 antibodies were minimal.

DISCUSSION

We have demonstrated that high titers of assembled, functional, full-length IgG1 antibody can be secreted by *A. niger*. Although there were previous reports of secretion of full-length antibody by yeast cells (11, 32), the titers attained were much lower than those that we report here. We observed higher titers of secreted trastuzumab (up to 0.9 g/liter) than of Hu1D10 (0.2 g/liter). The difference may arise because the codon usage in the DNAs encoding the variable regions of trastuzumab heavy and light chains was optimized for *A. niger*, whereas the mammalian DNA sequence was used for Hu1D10. It has been shown that codon optimization can lead to significantly higher expression in filamentous fungi (10, 27). Alternatively, the differences in the amino acid sequences of the variable regions of these two antibodies may affect production. We also demonstrated high titers of a secreted antibody fragment, Fab′.

Antibody light chains or heavy chains could be produced alone in *A. niger*. Wood et al. (32) were also able to detect separate secretion of light (λ) chains and heavy (μ) chains in *Saccharomyces cerevisiae*. In contrast, Takahashi et al. (25) were unable to detect secreted light or heavy (Fd fragment) chains when these were expressed individually in *P. pastoris*.

It has been repeatedly demonstrated that the secretion of foreign proteins by filamentous fungi is improved when the protein of interest is initially synthesized as a fusion to the C-terminus of a naturally secreted fungal protein (reviewed in reference 10). This is the system that we adopted in this work. An Fab fragment of an antibody was previously produced in *T. reesei* (19), and higher titers (up to 150 mg/liter) of the secreted fragment were obtained when the heavy chain was produced as a fusion with the native cellobiohydrolase I protein. However, in that study, the light chain was not produced as a fusion.

The production of a protein as a fusion requires a strategy for cleavage to release the desired protein with, preferably, an authentic N terminus. In this study, we relied on the native *Aspergillus* KexB protease to separate the fusion protein after a Lys-Arg sequence. However, this cleavage was not always efficient, especially for the light chain, possibly due to inaccessibility of the cleavage site caused by the secondary structure at the antibody N termini (24). Optimization of the amino acid sequence immediately next to the N-terminal side of the cleavage site improved cleavage to some extent. However, cleavage of 100% of the fusion proteins was not achieved, and the site of cleavage varied, so that heterogeneity at the N termini of the heavy and light chains resulted (Fig. 1). The insertion of three glycine residues at the C-terminal side of the cleavage site was required to result in complete cleavage without heterogeneity in the N termini of the released antibody chains. Insertion of other sequences at this location was not tested.

Assembly of antibody presumably occurs in the endoplasmic reticulum of *A. niger*, in the presence of chaperone proteins (e.g., BiP) and disulfide isomerases, as it does in mammalian cells. Cleavage by KexB would be expected to occur later, as the assembled antibody traverses the Golgi apparatus. It was clear that antibody assembly could occur despite the presence of glucoamylase at the N termini, because secreted antibody to which glucoamylase remained attached was observed in the culture supernatants of some transformants. Both trastuzumab and *Aspergillus*-derived Hu1D10 were shown to have antigen affinities similar to those of the equivalent antibodies produced in mammalian cell cultures. This was the case despite the presence of three glycines at the N terminus of An-3G-Hu1D10 or the Lys and Arg residues variably present at the N terminus of An-trast-I or An-Hu1D10.

A portion of the antibody heavy chain secreted by *Aspergillus* was not glycosylated, and an Asn residue was shown to be present at the consensus N-linked glycan attachment site (position 297). This finding suggests that the heavy chain had never been glycosylated, because deglycosylation with an enzyme with endoglycosidase H-type activity (EC 3.2.1.96) would leave a GlcNAc residue attached to Asn297, whereas deglycosylation with an enzyme with peptide: *N*-glycosidase F-type activity (EC 3.5.1.52) would convert the asparagine residue to an aspartate residue. Based on gel mobility data, the glycan on the heavy chain of the *Aspergillus*-derived antibody was approximately twice the size of that on the heavy chain of the control antibody produced in CHO cells. Wood et al. (32) showed that glycosylated and nonglycosylated forms of heavy chain μ were produced in yeast cells. Similarly, Horwitz et al. (11) showed that heavy chain γ produced in yeast cells appeared as a doublet on reducing SDS-PAGE, although the glycosylation states of the two heavy-chain forms were not determined. We were able to produce an aglycosylated mutant form of trastuzumab by changing the Asn residue at position 297 to Gln, but the titers of this form of the secreted antibody were reduced. Different substitutions at position 297 or at position 299 would also create aglycosylated heavy-chain variants and might allow higher titers of secreted antibody.

We examined the composition of glycan present on An-trast-I that had been purified from shake flask cultures with complex medium. The glycan on antibody purified from fermentor cultivations may differ from that obtained from defined media. In particular, the presence of terminal Galf may be dependent on the culture conditions and may also be affected by the strain of *A. niger* used (18). It has been suggested that terminal Galf may be antigenic (29); therefore, elimination of this residue from glycan by an appropriate choice of a strain or manipulation of a strain would be beneficial. Since approximately half of the heavy chains were shown to be nonglycosylated, it is likely that some fraction, conceivably a major fraction, of assembled antibodies had one nonglycosylated heavy chain and one glycosylated heavy chain. The glycan on IgG1 obtained from mammalian cells is believed to be sequestered between the two heavy chains and to engage in functionally important interactions with the protein. The size of the glycan may be restricted, so that complex glycans seldom include terminal sialic acid residues (reviewed in reference 14). It is possible that the glycan present on *Aspergillus*-derived IgG1, although larger than the mammalian glycan, also is sequestered between the heavy chains, since only one chain may be glycosylated. It was of interest to determine how the *Aspergillus* glycosylation pattern affects antibody function.

The long serum half-life of antibodies depends on their interaction with FcRn. It has been demonstrated that this interaction is not influenced by the structure of glycan on the

antibody Fc region and that even nonglycosylated antibody has a long serum half-life (26). However, rapid clearance could be caused by interactions with other receptors. For example, there is evidence that IgG1 with high-mannose-type glycosylation is cleared rapidly from serum possibly through interactions with mannan binding proteins or receptors (33). We saw no evidence of rapid clearance of An-trast-I from rat serum in our experiments. This result is consistent with the suggestion that although the glycan on *Aspergillus*-derived IgG1 is a large high-mannose-type structure, it is sequestered between the heavy chains.

The *Aspergillus*-derived Hu1D10 antibodies exhibited ADCC activity even though the structure of the attached carbohydrate was different from that for NS0-Hu1D10. However, it has yet to be determined whether the extents of ADCC activity were identical or slightly different for *Aspergillus*- and mammalian cell-derived IgG1 antibodies. Since the *Aspergillus*-derived Hu1D10 antibodies appear to be mixtures of aglycosylated and glycosylated forms and the former is expected to exhibit no ADCC activity, it will be interesting to isolate the glycosylated form and compare it to mammalian cell-derived Hu1D10 with regard to ADCC activity and binding to human CD16.

Horwitz et al. (11) also demonstrated that full-length antibody produced in yeast cells exhibited ADCC activity but not complement-dependent cytotoxicity (CDC), presumably because of inappropriate glycosylation. It is proposed that effective CDC function requires the presence of mammalian-type terminal sugars on the Fc glycan, whereas the presence of the core glycan sugars is sufficient to allow an interaction with FcγRIIIA on NK cells and for ADCC to function (reviewed in reference 14). We have not tested the CDC activity of *Aspergillus*-derived antibody, but it may be diminished relative to that of mammalian-derived antibody.

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REFERENCES

1. **Ballance, D. J., F. P. Buxton, and G. Turner.** 1983. Transformation of *Aspergillus nidulans* by the orotidine-5′-phosphate decarboxylase gene of *Neurospora crassa*. Biochem. Biophys. Res. Commun. **112:**284–289.
2. **Campbell, E. I., S. E. Unkles, J. A. Macro, C. van den Hondel, R. Contreras, and J. R. Kinghorn.** 1989. Improved transformation efficiency of *Aspergillus niger* using the homologous *niaD* gene for nitrate reductase. Curr. Genet. **16:**53–56.
3. **Carter, P., R. F. Kelley, M. L. Rodrigues, B. Snedecor, M. Covarrubias, M. D. Velligan, W. L. T. Wong, A. M. Rowland, C. E. Kotts, M. E. Carver, M. Yang, J. H. Bourell, H. M. Shepard, and D. Henner.** 1992. High level *Escherichia coli* expression and production of a bivalent humanized antibody fragment. Bio/Technology **10:**163–167.
4. **Carter, P., L. Presta, C. M. Gorman, J. B. B. Ridgway, D. Henner, W. L. T. Wong, A. M. Rowland, C. Kotts, M. E. Carver, and H. M. Shepard.** 1992. Humanization of an anti-p185^{HER2} antibody for human cancer therapy. Proc. Natl. Acad. Sci. USA **89:**4285–4289.
5. **Dorai, H., B. M. Mueller, R. A. Reisfeld, and S. D. Gillies.** 1991. Aglycosylated chimeric mouse/human IgG1 antibody retains some effector function. Hybridoma **10:**211–217.
6. **Eldin, P., M. E. Pauza, Y. Hieda, G. Lin, M. P. Murtaugh, P. R. Pentel, and C. A. Pennell.** 1997. High-level secretion of two antibody single chain Fv fragments by *Pichia pastoris*. J. Immunol. Methods **201:**67–75.
7. **Frenken, L. G. J., J. G. M. Hessing, C. A. M. J. J. van den Hondel, and C. T. Verrips.** 1998. Recent advances in the large-scale production of antibody fragments using lower eukaryotic microorganisms. Res. Immunol. **149:**589–599.
8. **Freyre, F. M., J. E. Vazquez, M. Ayala, L. Canaan-Haden, H. Bell, I. Rodriguez, A. Gonzalez, A. Cintado, and J. V. Gavilono.** 2000. Very high expression of an anti-carcinoembryonic antigen single chain Fv antibody fragment in the yeast *Pichia pastoris*. J. Biotechnol. **76:**157–163.
9. **Ghetie, V., J. G. Hubbard, J.-K. Kim, M.-F. Tsen, Y. Lee, and E. S. Ward.** 1996. Abnormally short serum half-lives of IgG in β2-microglobulin-deficient mice. Eur. J. Immunol. **26:**690–696.
10. **Gouka, R. J., P. J. Punt, and C. A. M. J. J. van den Hondel.** 1997. Efficient production of secreted proteins by *Aspergillus*: progress, limitations and prospects. Appl. Microbiol. Biotechnol. **47:**1–11.
11. **Horwitz, A. H., C. P. Chang, M. Better, K. E. Hellstrom, and R. R. Robinson.** 1988. Secretion of functional antibody and Fab fragment from yeast cells. Proc. Natl. Acad. Sci. USA **85:**8678–8682.
12. **Hudziak, R. M., G. D. Lewis, M. Winget, B. M. Fendly, H. M. Shepard, and A. Ullrich.** 1989. p185^{HER2} monoclonal antibody has antiproliferative effects in vitro and sensitizes human breast tumor cells to tumor necrosis factor. Mol. Cell. Biol. **9:**1165–1172.
13. **Jalving, R., P. J. I. van de Vondervoort, J. Visser, and P. J. Schaap.** 2000. Characterization of the kexin-like maturase of *Aspergillus niger*. Appl. Environ. Microbiol. **66:**363–368.
14. **Jefferis, R., J. Lund, and J. D. Pound.** 1998. IgG-Fc-mediated effector functions: molecular definition of interaction sites for effector ligands and the role of glycosylation. Immunol. Rev. **163:**59–76.
15. **Kostelny, S. A., B. K. Link, J. Y. Tso, M. Vasquez, B. H. Jorgensen, H. Wang, W. C. Hall, and G. J. Weiner.** 2001. Humanization and characterization of the anti-HLA-DR antibody 1D10. Int. J. Cancer **93:**556–565.
16. **Lange, S., J. Schmitt, and R. D. Schmid.** 2001. High-yield expression of the recombinant, atrazine-specific Fab fragment K411B by the methylotrophic yeast *Pichia pastoris*. J. Immunol. Methods **255:**103–114.
17. **Lund, J., N. Takahashi, J. D. Pound, M. Goodall, and R. Jefferis.** 1996. Multiple interactions of IgG with its core oligosaccharide can modulate recognition by complement and human Fcγ receptor I and influence the synthesis of its oligosaccharide chains. J. Immunol. **157:**4963–4969.
18. **Maras, M., I. van Die, R. Contreras, and C. A. M. J. J. van den Hondel.** 1999. Filamentous fungi as production organisms for glycoproteins of bio-medical interest. Glycoconjugate J. **16:**99–107.
19. **Nyyssonen, E., M. Penttila, A. Harkki, A. Saloheimo, J. K. C. Knowles, and S. Keranen.** 1993. Efficient production of antibody fragments by the filamentous fungus *Trichoderma reesei*. Bio/Technology **11:**591–595.
20. **Schwartz, W., D. Judd, M. Wysocki, L. Guerrier, E. Birck-Wilson, and E. Boschetti.** 2001. Comparison of hydrophobic charge induction chromatography with affinity chromatography on protein A for harvest and purification of antibodies. J. Chromatogr. A **908:**251–263.
21. **Shields, R. L., J. Lai, R. Keck, L. Y. O'Connell, K. Hong, Y. G. Meng, S. H. Weikert, and L. G. Presta.** 2002. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human FcγRIII and antibody-dependent cellular toxicity. J. Biol. Chem. **277:**26733–26740.
22. **Shinkawa, T., K. Nakamura, N. Yamane, E. Shoji-Hosaka, Y. Kanda, M. Sakurada, K. Uchida, H. Anazawa, M. Satoh, M. Yamasaki, N. Hanai, and K. Shitara.** 2003. The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. J. Biol. Chem. **278:**3466–3473.
23. **Simmons, L. C., D. Reilly, L. Klimowsky, T. S. Raju, G. Meng, P. Sims, K. Hong, R. L. Shields, L. A. Damico, P. Rancatore, and D. Yansura.** 2002. Expression of full-length immunoglobulins in *Escherichia coli*: rapid and efficient production of aglycosylated antibodies. J. Immunol. Methods **263:**133–147.
24. **Spencer, J. A., D. J. Jeenes, D. A. Mackenzie, D. T. Hayne, and D. B. Archer.** 1998. Determinants of the fidelity of processing glucoamylase-lysozyme fusions by *Aspergillus niger*. Eur. J. Biochem. **258:**107–112.
25. **Takahashi, K., Y. Toshifumi, T. Takai, C. Ra, K. Okumura, T. Yokota, and Y. Okumura.** 2000. Production of humanized Fab fragment against human high affinity IgE receptor in *Pichia pastoris*. Biosci. Biotechnol. Biochem. **64:**2138–2144.
26. **Tao, M.-H., and S. L. Morrison.** 1989. Role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. J. Immunol. **143:**2595–2601.
27. **Te'o, V. S. J., A. E. Czifersky, P. L. Bergquist, and K. M. H. Nevalainen.** 2000. Codon optimization of xylanase gene *xynB* from the thermophilic bacterium *Dictyoglomus thermophilum* for expression in the filamentous fungus *Trichoderma reesei*. FEMS Microbiol. Lett. **190:**13–19.
28. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
29. **Wallis, G. L. F., R. L. Easton, K. Jolly, F. W. Hemming, and J. F. Peberdy.** 2001. Galactofuranoic-oligomannose N-linked glycans of α-galactosidase A from *Aspergillus niger*. Eur. J. Biochem. **268:**4134–4143.

30. **Ward, M., L. J. Wilson, and K. H. Kodama.** 1993. Use of *Aspergillus* overproducing mutants, cured of integrated plasmid, to overproduce heterologous proteins. Appl. Microbiol. Biotechnol. **39:**738–743.
31. **Ward, M., L. J. Wilson, K. H. Kodama, M. W. Rey, and R. M. Berka.** 1990. Improved production of chymosin in *Aspergillus* by expression as a glucoamylase-chymosin fusion. Bio/Technology **8:**435–440.
32. **Wood, C. R., M. A. Boss, J. H. Kenten, J. E. Calvert, N. A. Roberts, and J. S. Emtage.** 1985. The synthesis and in vivo assembly of functional antibodies in yeast. Nature **314:**446–449.
33. **Wright, A., and S. L. Morrison.** 1994. Effect of altered C_H2-associated carbohydrate structure on the functional properties and in vivo fate of chimeric mouse-human immunoglobulin G1. J. Exp. Med. **180:**1087–1096.