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Characterization of a recombinant humanized anti-cocaine monoclonal antibody produced from multiple clones for the selection of a master cell bank candidate

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ABSTRACT

We have generated a humanized anti-cocaine monoclonal antibody (mAb), which is at an advanced stage of pre-clinical development. We report here *in vitro* binding affinity studies, and *in vivo* pharmacokinetic and efficacy studies of the recombinant mAb. The overall aim was to characterize the recombinant antibody from each of the three highest producing transfected clones and to select one to establish a master cell bank. In mAb pharmacokinetic studies, after injection with h2E2 (120 mg/kg iv) blood was collected from the tail tip of mice over 28 days. Antibody concentrations were quantified using ELISA. The h2E2 concentration as a function of time was fit using a two-compartment pharmacokinetic model. To test *in vivo* efficacy, mice were injected with h2E2 (120 mg/kg iv), then one hour later injected with an equimolar dose of cocaine. Blood and brain were collected 5 min after cocaine administration. Cocaine concentrations were quantified using LC/MS. The affinity of the antibody for cocaine was determined using a [^{3}H] cocaine binding assay. All three antibodies had long elimination half-lives, 2–5 nM Kd for cocaine, and prevented cocaine's entry into the brain by sequestering it in the plasma. Pharmacokinetic and radioligand binding assays supported designation of the highest producing clone 85 as the master cell bank candidate. Overall, the recombinant h2E2 showed favorable binding properties, pharmacokinetics, and *in vivo* efficacy.

1. Introduction

Immunotherapy has shown potential as a treatment for cocaine abuse. Anti-cocaine antibodies have been shown to bind to cocaine, preventing its entry into the brain [1]. In clinical trials, active immunization with an anti-cocaine vaccine induced the production of anti-cocaine antibodies [3,4]. However, effectiveness in inhibiting cocaine use was variable, likely due to the inability to control antibody titers. In the first trial, some level of effectiveness was observed that depended on the level of antibody titers raised. The minimum effective plasma concentration of anti-cocaine antibodies was achieved in only a subpopulation of subjects. However, in a subsequent larger trial, though some patients generated levels of anti-cocaine antibodies proposed as adequate, no significant effect on cocaine usage was observed. An alternative immunotherapy using passive immunization with an anti-cocaine monoclonal antibody should provide a more consistent clinical response, as the dose can be directly controlled and the affinity of the antibody is constant and known.

The recombinant humanized anti-cocaine antibody, h2E2, has previously been reported to have high affinity and selectivity for cocaine over its inactive metabolites [5,6]. It is able to prevent cocaine entry into the brain in rats [5], and cocaine's active metabolite, cocaethylene, entry into the brain in mice [7]. It has a long half-life in both rats and mice [5,7]. This mAb also increased the amount of cocaine needed to reinstate self-administration behavior in a rat model of relapse by three-fold [8], which should translate into a decrease in the probability of cocaine induced relapse. On the basis of these findings h2E2 is a lead candidate as an immunotherapeutic for cocaine abuse.

The recombinant h2E2 mAb is currently being produced in large

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(g/L) quantities from transfected Chinese Hamster Ovary (CHO) clones [5]. In order to advance h2E2 toward clinical trials, it is necessary to establish a master cell bank to permit the production of protein suitable for use in humans [9]. The selection of a single clone is a critical step in establishing this cell bank [10]. It was shown, using the same three mAb samples as in the current study, that post-translational modifications (glycosylation) can vary between h2E2 produced by different clones (cell lines) with different production yields [11]. The effects of these differences in glycosylation on the pharmacokinetics of monoclonal antibodies are unclear since contradictory results have been obtained in previous studies [12]. A recent review by Higel et al. [12] discusses how increasing levels of high mannose glycans can decrease the pharmacokinetic half-life of antibodies, whereas other types of N-glycosylation have been found to have the opposite effect. This review concluded that high mannose glycan levels influence the pharmacokinetics of IgGs by increasing the clearance rate via the mannose receptor, which is highly expressed on immune cells. For other N-glycans and glycoforms the results were less clear [12]. With respect to our h2E2 mAb, Kirley et al. did not show any difference in the mannose levels in the clones, however, there were differences observed in some neutral biantennary N-glycan levels, especially in G_{0F} in antibody from clone 85 when compared to that from clones 188 and 323 [11]. Although there is no reason to believe that this heterogeneity of glycosylation of h2E2 should affect the affinity for cocaine, it is important to characterize the h2E2 protein produced from potential clones for *in vitro* and *in vivo* efficacy before selecting a clone to establish the master cell bank.

It is generally assumed that an ideal anti-drug antibody used for the prevention of relapse should have a high affinity for its target, a long biological half-life, and be able to rapidly alter the pharmacokinetics of the target drug [1]. This can be tested by *in vitro* binding studies to determine the Kd (a measure of binding affinity) of the antibody for its target, an *in vivo* pharmacokinetic study to determine the half-life, and an *in vivo* study of the tissue distribution of the target drug, particularly to the brain, in the presence of the antibody. Combined, this battery of tests can provide confidence that the antibody has appropriate pharmacokinetic (PK) and pharmacodynamic (PD) properties. Therefore, material from the top three producing h2E2 clones were screened using the described battery of tests in order to determine which of these clones would be appropriate candidates to establish a master cell bank.

2. Materials and methods

2.1. Antibody production and preparation

A population of h2E2-producing clones was generated by Catalent Pharma Solutions (Madison,WI) using their GPEx technology [13] to stably transfect Chinese Hamster Ovary (CHO) cells with multiple copies of the cDNAs for the heavy and light chains of h2E2. The top three producing clones (85, 188 and 323) were selected for further optimization of h2E2 production levels. The clones were passaged every 3–4 days during the exponential phase, maintaining a viability of 90% or better in either CD OptiCHO (Life Technologies), PowerCHO-2 (Lonza) or G12.1 (Lonza) growth media. Cells were inoculated at a cell density of 300,000 cells/mL in each media and incubated in a Multitron shaking incubator. To find the optimal growth conditions, three different culture conditions using the feed supplements Efficient C, F12.2 or F12.7 were performed in each growth media for each clone. Additional feed supplements including Cell Boost 4 PS307, L-glutamine, and glucose were included in all growth flasks. Duplicate 500 mL capacity flasks (190 mL total volume) were inoculated for each condition. The duplicate shaker flasks for each set of conditions were sampled on alternate days and were harvested when viabilities were approximately 50%, up to a maximum of 20 days. The secreted recombinant h2E2 mAb was purified using a series of filtrations and chromatography purifications using protein A. For animal studies, the purified h2E2 was tested for sterility to ensure low virus and endotoxin levels. To decrease the injection volume, the h2E2 was concentrated using size-exclusion filtration concentration with a 3000 molecular weight cutoff. Final protein concentrations were determined by 280 nm absorbance.

2.2. [^{3}H]Cocaine-binding assay

Cocaine binding studies were carried out by immunoprecipitation of h2E2 bound to [^{3}H]cocaine as previously described by Norman et al. [5], except that the incubations were done at 4 °C instead of at room temperature, and all reagents were added at once instead of sequential additions and incubations. In summary, a fixed concentration (0.4 nM) of h2E2 mAb was incubated with a serially diluted range of [^{3}H]cocaine concentrations (0.5–100 nM) with the specific activity diluted with unlabeled cocaine to 1 μCi/200 pmole. A goat anti-human antibody (4 nM) and a rabbit anti-goat antibody (40 nM) were added and the samples were incubated overnight (18–20 h) at 4 °C. Bound ligand was separated by filtration through Whatman GF/F filters using a Brandel Harvester and a single wash with 2 mL of cold PBS. Filters were placed in scintillation fluid and radioactivity was measured using a Beckman Coulter LS6500 Multi-Purpose Scintillation Counter. Non-specific binding was determined by the counts measured in the absence of h2E2. Specific binding was calculated by subtracting the non-specific counts from counts obtained from samples containing the h2E2. CPM specifically bound as a function of total cocaine concentration was fit to a saturation binding curve to determine Kd and Bmax. Curves displayed on the figures were generated by taking the mean of all the replicate curve fits. Both fitted parameters (Kd and Bmax) from at least three sets of experiments were statistically compared using a one-way ANOVA.

2.3. Animals

All studies were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* [14] and under a protocol approved by the Institutional Animal Care and Use Committee at the University of Cincinnati. Jugular vein catheterized Swiss-Webster mice (19–22 g) were purchased from Taconic Farms. Mice were housed individually on a 14/10- hour light/dark schedule with unrestricted access to food and water.

2.4. Pharmacokinetics of h2E2 mAb in mice

Three groups of mice were injected with h2E2 produced from one of the three clones (n = 6 mice per clone) at a dose of 120 mg/kg i.v. At times from 7 min to 28 days, 10 μL of blood was collected from a small incision at the tip of the tail. Blood was immediately diluted into 90 μL of citrate buffer (0.1 M, pH 4) and stored at 4 °C. Within a week of collection, blood was further diluted for storage at 4 °C at a final dilution of 1:100 in tris-buffered saline pH 7.2 containing BSA (0.5%).

Blood h2E2 concentrations were quantified using ELISA as described previously [15]. Briefly, h2E2 was captured by binding to a benzoylecgonine 1,4-butanediamine-BSA (BE-BSA) conjugate adsorbed onto 96 well plates, which were then blocked using BSA (0.5%). The bound h2E2 was detected by incubation with biotinylated goat anti-human polyclonal antibodies and the colorimetric signal was generated using a streptavidin-alkaline phosphatase hydrolysis of 4-nitrophenyl phosphate di (tris) salt.

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