Monoclonal Antibodies which Preferentially Bind to 22 K Human Growth Hormone Rather than Its 20 K Variant

TOHRU NAKANISHI, HIROSHI MATSUI AND HIROSHI NOGUCHI

Abstract

We have established 13 hybridoma cell lines which secrete mouse IgG1 monoclonal antibodies (McAbs) to human growth hormone (hGH). Binding affinity and binding specificity of McAbs were analyzed by competitive radioimmunoassay. Among these McAbs, CL. B1 showed a high affinity of \(9.8 \times 10^8\) \(\text{mol}^{-1}\), and all McAbs so far tested showed very weak cross-reactivity or none at all with human prolactin (hPRL) and human chorionic somatomammatropin (hCS; human placental lactogen). Analysis of binding sites of McAbs using hGH variant and fragments in both ELISA and RIA demonstrated that McAbs could be classified into two groups. All the McAbs obtained in this study bound to plasmin-digested fragment S2 (hGH 1–134 and 141–191) and fragment α3 (hGH 1–134 and 147–191). However, five (such as 1D2) out of 13 McAbs bound to fragment F1 (hGH 1–134) and others (such as CL. B1) did not. The McAb CL. B1 in the latter group showed low affinity with 20 K hGH (residue 32–46 deleted in native 22 K hGH) in contrast to high affinity with hGH (22 K). This suggests that the former McAbs recognize an epitope located at the N-terminal two-third part of hGH. In contrast, the McAbs of the latter group are likely to recognize three-dimensional structure of native 22 K hGH.

Human growth hormone (hGH, Somatomropin) is one of growth hormones which are synthesized in the anterior lobe of the pituitary gland and has a major action in promoting somatic growth. It is a protein of M. W. 22,000 daltons (referred to as 22K hGH), consisting of a single polypeptide chain of 191 amino acids with two disulfide cross-linking bridges (Wallis, 1978).

There are two other hGH-related hormones, chorionic somatomammatropin (hCS; placental lactogen) and human prolactin (hPRL), which have been classified into the same family as hGH, and hGH shares 85% of amino acid residues with hCS but only 16% with hPRL (reviewed by Wallis, 1978).

The assay of hGH is important in clinics in diagnosing hyperpituitarism, acromegary and hGH deficiency (dwarfism). The level of hGH in serum has been routinely measured by radioimmunoassay (RIA) and enzyme immunoassay (EIA) using rabbit antiserum

Received January 9, 1989
Address to which correspondence should be directed: HIROSHI NOGUCHI, Ph. D. Laboratory of Biotechnology, Takarazuka Research Center, Sumitomo Chemical Co. Ltd., Takarazuka, Hyogo, 665 Japan.
to hGH. From the homology of amino acid residues among hGH, hCS and hPRL, anti-hGH serum would be expected to cross-react with hCS at a high rate, but not with hPRL. In practice, however, a strong cross-reaction between hGH and hPRL has been observed at a relatively high frequency because the hGH preparation for immunization has often been contaminated with hPRL (Hwang et al., 1971). An antibody with a high specificity should be applied to clinical diagnosis of hGH in order to accurately measure the level of hGH in body fluid.

Furthermore, recent studies showed that there is a native variant of hGH in human serum or in the anterior lobe of the pituitary gland (Lewis et al., 1978, 1980). It is composed of 176 amino acids and referred to as 20K hGH. Although 20K hGH lacks the early insulin-like and intrinsic diabetogenic activities of 22K hGH, it shows the same order of growth promoting activity as 22K hGH (Lewis et al., 1980). In this point, the selective assay of 22K hGH discriminating it from 20K hGH would be important in diagnostics and basic research on this hormone.

A hybridoma technique established by Köhler and Milstein (1975) made it possible to get antibodies with high specificity. We have established hybridoma cell lines producing mouse monoclonal antibodies (McAbs) which each recognize at least two different epitopes on the hGH molecule with high specificity, and one group of McAbs was found to recognize selectively 22K hGH when compared with 20K hGH in its binding affinity. In this paper, we report the results of the analysis of the binding specificity, binding affinity and binding site determination of these McAbs.

**Materials and Methods**

**Hormones**

hGH purified from pituitaries, Somatropin (Kabi Vitrum AB, Stockholm, Sweden), was used for immunization and screening. Methionyl recombinant hGH, Somatorem (Kabi Vitrum AB), was also used for binding study. Fragments and variants of hGH (see Fig. 2 A) were prepared by Dr. U. J. Lewis, The Whittier Institute for Diabetes and Endocrinology, La Jolla, CA and also purchased from Funakoshi Ltd. (Tokyo, Japan). Human prolactin (hPRL) was purchased from Calbiochem-Behring Corp. (La Jolla, CA). Human chorionic somatomamotropin (hCS) was from Pharmacia Fine Chemicals AB (Uppsala, Sweden).

**Immunization**

Five-weeks old female BALB/c mice (Nihon Clea Inc., Tokyo, Japan) were intraperitoneally immunized four times at 3 weeks intervals with 100 µg hGH with complete Freund’s adjuvant (Difco Laboratories, Detroit, Michigan) for priming and with incomplete Freund’s adjuvant (Difco Laboratories) for booster. Four days after the last intravenous injection with hGH in saline, the spleen was removed.

**Myeloma cells, cell fusion and cloning of hybrids**

Spleen cells were fused with mouse myeloma cells P3X63Ag8U1 (P3U1, Marguillies et al., 1976) which were kindly provided by Dr. Watanabe, Kyushu University, Fukuoka, Japan and Dr. Maruyama, Osaka University School of Medicine, Osaka, Japan, according to the technique of Watanabe et al. (1978). Spleen cells (10⁶ cells) were fused with P3U1 myeloma cells (10⁵ cells) in the presence of 45% polyethylene glycol (PEG) 6,000 (Sigma Chemical Co., St. Louis, MC) in Eagle’s MEM (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan). After incubation for 8 min at room temperature, the cells were gradually diluted with 16 ml of Eagle’s (2 ml/min) followed by further addition of 17 ml Eagle’s MEM and washed by centrifugation at 1,000 rpm for 7 min at 4°C. The cells were then suspended in 150 ml of RPMI 1640 medium (Research Institute for Microbial Diseases) supplemented with 10% fetal calf serum (Gibco, Chagrin Falls, Ohio) and 50 µM 2-mercaptoethanol (Nakarai Chemicals Ltd., Kyoto, Japan). Aliquots (0.2 ml/well) were distributed into 96-well microplates (Sumitomo Bakelite Co. Ltd., Tokyo, Japan) at a cell density of 1.2 × 10⁴ cells per well as myeloma
cells and were cultured at 37°C in an atmosphere of 5% CO₂. The hybrids were grown in selective HAT medium (Littlefield, 1964) containing 10⁻⁴ M hypoxanthine (Sigma Chemical Co.), 4 x 10⁻⁷ M aminopterin (Sigma Chemical Co.) and 1.6 x 10⁻⁵ M thymidine (Sigma Chemical Co.). The hybrids were cloned by limiting dilution.

**Screening**

Screening was done 10-12 days after fusion and also 10-12 days after cloning by means of modified ELISA methods (Engvall and Perlmann, 1972). Briefly, hGH (100 ng/well) dissolved in PBS (pH 7.2) was adsorbed to polyvinylchloride 96-well microplate (Falcon Microtest III flexible assay plate, Becton Dickinson, Oxnard, CA) and then blocked with 1% (w/v) BSA in PBS (pH 7.2). Culture media of hybrids and alkaline phosphatase (ALP) labelled anti-mouse immunoglobulin antibody (New England Nuclear, Boston, MA) with 1% (w/v) BSA in PBST (0.05% (w/v) Tween-20 in PBS PH 7.2) were added to the plate sequentially. The reaction was quantitated by adding substrate of p-nitrophenylphosphate disodium and reading the optical density at 405nm in a Titertek Multiskan automated spectrophotometer MCC (Flow Laboratories, McLean Virginia).

**Iodination of hGH, its variants and fragments**

Iodination of hGH, its variants and fragments were performed by using Bolton-Hunter reagent (Amersham International plc., Buckinghamshire, England). A 40 μl volume of Bolton-Hunter reagent was added to an Eppendorf tube (Eppendorf Geratebau, Hamburg, Fed. Rep. of Germany) and dried with N₂ air. Then 2 μg of hormones in 20 μl of 0.1 M borate buffer (pH 8.5) was added to the tube and incubated for 20 min at 0°C. After incubation, 0.5 ml of 0.1 M borate buffer (pH 8.5) containing 0.2 M glycine was added to the mixture which was further incubated for 10 min at 0°C. Finally, the mixture was applied to the column (PD-10, Pharmacia Fine Chemicals) and eluted with 50 mM phosphate buffer (pH 7.5) containing 0.25% gelatin. The radioactivity of each fraction was counted and the peak fractions were used for subsequent assays.

**Class and subclass determination**

The class and subclass of McAbs were determined by modified RIA methods. Briefly, McAbs were incubated with ¹²⁵I-hGH followed by incubation with anti-mouse Ig (anti-class and anti-subclass) serum (Miles Laboratories Inc., Naperville, ILL.). Radioactivity of precipitates was measured with a γ-counter (Beckman Instruments Inc., Fullerton, CA).

**Binding specificity and binding affinity test**

Culture supernatants or ascitic fluids were assayed by a RIA method. A 50 μl volume of diluted culture supernatant or ascitic fluid and 50 μl of ¹²⁵I-hGH (50 μCi/μg, 10,000–20,000 cpm) were incubated at 4°C overnight and further incubated at 37°C for 2 hr. Then 50 μl of anti-mouse Ig (Miles) and 10 μl of 1% normal mouse serum were added. Following incubation at 37°C for 2 hr, precipitates were separated by centrifugation at 3,000 rpm for 30 min and radioactivity was measured. To determine binding specificity, a simultaneous competition method was used; that is, 10 μl of unlabeled polypeptides (hGH, its fragment, hCS and hPRL) and 50 μl of ¹²⁵I-hGH were added simultaneously to a diluted solution of McAb and processed as described above. The binding constant was calculated by Scatchard plot analysis (Scatchard, 1949).

**Binding site determination**

The binding site recognized by McAbs was determined by an ELISA method. Briefly, Falcon Microtest III flexible plates were coated with 1 or 4 μg/ml hGH or its variants dissolved in PBS (pH 7.2). After being blocked with 1% BSA in PBS, they were incubated with appropriately diluted culture supernatant or ascitic fluid, followed by incubation with ALP labelled anti-mouse Ig (NEN). The reaction was quantitated by adding the substrate and reading the optical density at 405 nm in a Titertek Multiskan automated spectrophotometer.

**Results**

**Cell fusion**

In successful fusions, colony growth of hybrids was observed in 100% wells of a 96-well microplate used and a positive anti-hGH antibody titre (more than 5–10 fold
higher radioactivity in $^{125}$I-hGH co-precipitated than that of background in the RIA system) was detected in 35% of a total of 170 wells. Then cells in the antibody titre-positive wells were cloned and finally 13 stable hybridomas were established. The subclass of 13 McAbs was all IgG1 (Table 1). Nine of 13 hybridoma cell lines gave a high antibody titre demonstrating 50% binding of the maximum $^{125}$I-hGH binding with McAb at 1–30×10⁴ fold dilution of ascitic fluid derived from hybridomas (Table 1).

### Binding affinity of McAbs

The binding affinity of McAbs was tested by determining the concentration of unlabeled hGH needed to compete with $^{125}$I-hGH in binding with McAbs (Table 1). The amount of unlabeled hGH (Cl₅₀) required for 50% inhibition in simultaneous competition was widely varied and that of CL.B1 was the lowest (5 ng/well), showing the highest affinity with hGH among McAbs obtained in this experiment. This was confirmed by calculating the affinity constant value (Ka) of McAb according to a Scatchard plot (Scatchard, 1949). Ka values for 4 McAbs tested varied from 2.0×10⁸ to 9.6×10⁸ 1/mol (Fig. 1 and Table 1). CL.B1 McAb showed the highest Ka value.

### Binding specificity of McAbs

The specificity of McAbs was tested by a competition experiment as described in Methods. The inhibition percent by unlabeled hPRL was determined in a heterologous competition system. Unlabeled hPRL at the same concentration as that of unlabeled hGH, which gave 50% inhibition (Cl₅₀) of the maximum $^{125}$I-hGH binding with McAb in homologous competition system, was used. All four McAbs showed only weak binding (at most 8% inhibition).

<table>
<thead>
<tr>
<th>McAb</th>
<th>Class Subclass</th>
<th>Titreᵃ</th>
<th>Cl₅₀hGHᵇ (ng/well)</th>
<th>Cross reactivity with hPRLᶜ (% inhibition)</th>
<th>Affinityᵈ (1/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL. B1</td>
<td>IgG1</td>
<td>280000</td>
<td>5</td>
<td>8</td>
<td>9.6×10⁸</td>
</tr>
<tr>
<td>B1</td>
<td>IgG1</td>
<td>10000</td>
<td>40</td>
<td>8</td>
<td>2.0×10⁸</td>
</tr>
<tr>
<td>C1</td>
<td>IgG1</td>
<td>1500</td>
<td>40</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>C5</td>
<td>IgG1</td>
<td>250000</td>
<td>9</td>
<td>8</td>
<td>5.0×10⁸</td>
</tr>
<tr>
<td>1B6</td>
<td>IgG1</td>
<td>80000</td>
<td>35</td>
<td>NDᵇ</td>
<td>ND</td>
</tr>
<tr>
<td>1C2</td>
<td>IgG1</td>
<td>25000</td>
<td>38</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1C9</td>
<td>IgG1</td>
<td>10000</td>
<td>102</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1D2</td>
<td>IgG1</td>
<td>6000</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1D7</td>
<td>IgG1</td>
<td>38000</td>
<td>25</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2A3</td>
<td>IgG1</td>
<td>800</td>
<td>37</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2A6</td>
<td>IgG1</td>
<td>320000</td>
<td>25</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2C4</td>
<td>IgG1</td>
<td>250000</td>
<td>45</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2E5</td>
<td>IgG1</td>
<td>150000</td>
<td>60</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

RIAs was used for all experiments.

ᵃDilution fold of ascites giving 50% of the maximum $^{125}$I-hGH binding to McAbs

ᵇThe amount of unlabeled hGH per well giving 50% inhibition of the maximum $^{125}$I-hGH binding to McAbs in simultaneous competition system

ᶜInhibition percent by unlabeled hPRL at the same concentration as Cl₅₀ of hGHᵇ in $^{125}$I-hGH binding to McAbs (heterologous competition system)

ᵈAffinity constant against hGH determined by Scatchard plot

ᵇNot done
Vol. 36, No. 4 MONOCLONAL ANTIBODIES TO GROWTH HORMONE

485

Fig. 1. Scatchard plot analysis on binding of McAbs to native 22K hGH. Simultaneous competition of unlabeled hGH with $^{125}$I-hGH was done to determine binding affinity. $K_a$ was calculated by the method of Scatchard (1949). McAbs are as follows; (●) B1, (○) C5, (▲) 1D2, (△) CL. B1.

to hPRL at a concentration corresponding to Cl₅₀ of hGH. McAb C1 did not cross-react with hPRL even if 40 ng of hPRL per well was added in a competitive binding system.

Four McAbs also showed weak binding to hCS or none at all, but they bound to methionyl recombinant hGH at the same rate as did to native hGH (data not shown).

**Binding site recognized by McAbs**

The binding site was determined by ELISA using hGH variant and fragments (Fig. 2B). All McAbs bound to plasmin-digested hGH fragment S2 (hGH 1-134 and 141-191) and fragment α3 (hGH 1-134 and 147-191) as well as to native hGH. However, McAbs were divided into two groups when the binding of McAbs to F1 fragment (hGH 1-134) was examined. One group (B1, C1, C5, 1C9 and 1D2) bound to F1 fragment, but the other (CL.B1, 2A6, 1D7 and others) did not. This suggests that the epitope on the hGH molecule to which the former group of McAbs such as 1D2 bind is largely located within the N-terminal two-third part of hGH. In ELISA competition experiments using hGH coated PVC plates (each of unlabeled hGH fragments was simultaneously added to hGH coated PVC plates with the former group of McAb and the bound amount of McAb was estimated), fragments S2, α3 and native hGH clearly competed with $^{125}$I-hGH binding to McAb, but fragment F1 showed only slight competition in this system (data not shown). The affinity of McAb 1D2 with fragment F1 was estimated with $^{125}$I-F1 fragment in RIA system. The $K_a$ value for McAb 1D2 to F1 fragment was calculated to be $3.0 \times 10^6$ 1/mol (Fig. 3), being one hundredth that of this McAb to native hGH. From this result, McAb 1D2 (the former group of McAbs) seemed to weakly bind to fragment F1.

Next, the binding of McAbs to the C-terminal one-third part of hGH (this fragment is called F2) was examined. In both
Fig. 2 A. Structure of variants, fragments and native form of hGH. Solid lines show residues of hGH. Numbers below the line indicate the amino acid sequence numbers corresponding to those of native 22K hGH. Dotted lines show disulfide linkages of hGH molecule.

B. Binding assay of McAbs to native form and variants of hGH. ELISA system was
EIA and RIA systems, neither of the latter McAbs (for example, CL.B1) or the former McAbs showed any binding to F2 fragment. As shown in Fig. 3, McAb CL.B1 also showed a high affinity with $K_a = 9.6 \times 10^8$ l/mol to native hGH. In contrast, its $K_a$ to 20K variant was almost as low ($3.7 \times 10^7$ l/mol) as the $K_a$ of McAb 1D2 to 20K ($3.6 \times 10^7$ l/mol). $K_a$ ratios ($K_a$ to native hGH/$K_a$ to 20K variant) of these McAbs were 26 for CL.B1 and 7 for 1D2, respectively.

**Discussion**

We have established 13 hybridomas which secrete McAbs to hGH. All McAbs belonged to IgG1. Hybridoma cells produced a large amount of McAbs in culture supernatant *in vitro* (10 to 100 µg IgG/10^6 cells/day) and in ascitic fluid of mice (5 to 50 mg/ml). In competitive RIA, McAb of CL.B1 gave the lowest $C_{50}$ value and the highest affinity constant to hGH ($K_a = 9.8 \times$ used for this assay. Microtest plates were coated with 1 µg/ml (a3) or 4 µg/ml (F1, S2 and native) of polypeptides for 2 hr at 37°C. Diluted ascitic fluids (which showed 50% of maximam binding in ELISA system) were added to the plates and incubated for 30 min at 37°C. Then the plates were washed and alkaline phosphatase-labeled anti-mouse Ig (diluted for 1,000 fold) was added to the plates. The plates were further incubated for 1 hr at 37°C. Finally, the plates were washed and substrates were added. McAbs are indicated at the bottom of the figure. My. means ascitic fluid obtained from mice injected with myeloms cells.
10^8 1/mol). All McAbs were shown to bind only weakly to hPRL and hCS or not at all, indicating high specificity.

Hybridoma cells which secrete anti-hGH McAbs have been established by several other groups so far (Bundesen et al., 1980; Ivanyi and Davis, 1980; Jonsdottir et al., 1981, 1983; Retegui et al., 1982). They characterized the binding affinity and binding specificity (cross reactivity) of McAbs. However, little has been studied about the binding sites recognized by the McAbs. In this paper, we have examined specific binding sites using fragments and variants of hGH and have classified at least two groups of McAbs recognizing different epitopes of hGH.

Five out of 13 McAbs, such as 1D2, bound to fragment F1, indicating that these McAbs would mainly recognize an epitope located at the N-terminal two-third part of hGH. In contrast, the other McAbs such as CL.B1 did not bind to either F1 or F2 although CL.B1 bound to S2 and α3 variants as well as native hGH at high affinity. The binding affinity of CL.B1 to 20K hGH variant was one twenty-sixth of that of CL.B1 to 22K hGH. In addition, 1D2 (the former group) slightly bound to reduced and alkylated 22K hGH, but CL.B1 (the latter group) did not bind at all in Western blotting (data not shown). A line of evidence strongly suggests that CL.B1 would recognize the three-dimensional structure of hGH rather than a sequence of amino acid residues in the C-terminal one-third part of hGH.

Recently, a model three-dimensional structure of hGH was proposed (Cohen and Kuntz, 1987), and confirmed by using genetically engineered variants of porcine growth hormone (Abdel-Meguid et al., 1987), hGH being composed of four α-helix domains. Amino acid residues 32-46 of 22K hGH which are deleted in 20K hGH are likely to be located between the first N-terminal α-helix and second α-helix. In addition, the cleavage point of hGH into fragments F1 and F2 seems to be located in the region between the third and forth α-helices. Both positions (amino acid residues 32-46 and 134-135) are supposed to be located near each other. McAbs CL.B1 might recognize nearby three-dimensional sites of the two positions at the same time, because CL.B1 did not bind to either F1 or F2 fragment and the binding affinity with 20K hGH was decreased 26 times comparing with that to 22K hGH. If CL.B1 recognizes a sequence of amino acid residues in F2 fragment, it might show the same binding affinity with 20K as 22K hGH because both 20K and 22K hGH contain identical amino acid sequences in F2. An alternative explanation for the low affinity of CL.B1 with 20K hGH is that the deletion of amino acid residues 32-46 (20K hGH) in contrast to the deletion of amino acid residues 135-146 (α3) might drastically change the three-dimensional structure of hGH, resulting in lowering the affinity of CL.B1 with 20K hGH in contrast to the high binding of CL.B1 to α3 fragment.

As is well known, McAbs are superior to conventional antisera as to homogeneity, specificity and the amount of antibody to be obtained. The McAbs to hGH would be widely used for quantitative assays of hGH as well as in physicochemical studies on the structure of hGH. In addition, two groups of McAbs recognizing different epitopes on the hGH molecule which we report here would be useful for application to a one step sandwich assay of hGH. Furthermore, McAb CL.B1 could be applied for estimating the precise amount of native 22K hGH in a body fluid sample, being differentiatied from 20K hGH by its different affinity. 20K hGH has growth-promoting activity quite similar to that of 22K hGH, but it is not diabetogenic and lacks the early insulin-like properties of 22K hGH (hypoglycemia and decrease in serum free fatty acids) (Lewis et al., 1980). Differential
assays of 22K hGH discriminating it from 20K hGH would be valuable in determining the real physiological roles of the two hormones and in understanding the association of hGH's diabetogenic activity with diabetic diseases such as diabetic retinopathy.

Finally, McAb CL.B1 with high affinity with hGH \((9.8 \times 10^8)\) has been used for constructing an EIA kit which gives 10 to 200-fold higher sensitivity than conventional hGH diagnosis kits, its lower assay limit being 0.1 pg hGH per tube and making it possible to determine a physiological hGH level in urine as well as in serum and plasma without the induction of hGH secretion by pharmacological drugs (Hashida et al., 1988).

Acknowledgements

We thank Drs T. Tsushima and U. J. Lewis for kindly giving the variant and fragments of hGH which were used in this study.

References


