Monoclonal antibodies to growth hormone (GH) prolong liver GH binding and GH-induced IGF-I/IGFBP-3 synthesis

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Monoclonal antibodies to growth hormone (GH) prolong liver GH binding and GH-induced IGF-I/IGFBP-3 synthesis. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E308–E315, 1999.—This time-course study further explored the mechanisms whereby monoclonal antibodies (MAbs) may enhance growth hormone (GH) effects. Hypophysectomized rats were killed 0, 1, 3, 6, 12, 24, and 48 h after a single injection of bovine (b) GH alone or complexed with an anti-bGH MAb. Serum insulin-like growth factor I (IGF-I) and liver IGF-I synthesis and an enhanced induction of IGFBP-3. In the presence of high doses of MAb (20). This increase was observed 18 h after a single injection of MAb-GH complexes and might have resulted from a greater occupancy of GH receptors, from a prolongation of GH binding to its receptors, or from both mechanisms together.

On the other hand, the action of insulin-like growth factor (IGF-I) is strongly regulated by multiple IGF-binding proteins (IGFBPs) (27). Most of the IGF-I in serum circulates as a 150-kDa complex consisting of IGF-I, IGFBP-3, and a non-IGF-I binding component termed acid-labile subunit (2). Formation of this large complex alters IGF-I distribution and clearance and modifies IGF-I bioactivity (15). The GH dependence of IGFBP-3 was inferred from the decreased levels in hypophysectomized subjects and GH-deficient children and from the increased levels in acromegalic patients (2, 4). Also, GH administration to hypophysectomized rats was reported to increase plasma IGFBP-3 levels (33). Therefore, modification of IGFBP levels in response to injection of MAb-GH complexes may also occur and contribute to potentiation of GH growth-promoting actions.

To further explore the mechanisms of the MAb-induced potentiation of GH effects, we investigated in the present study the kinetics of hepatic GH binding and serum IGFBP levels in response to MAb-GH complexes in hypophysectomized rats.

MATERIALS AND METHODS

Animals. Female Wistar rats were hypophysectomized at 4 wk of age (body weight: 70.0 ± 0.1 g) (IFFA CREDO, Lyon, France) and delivered to our animal quarters 1 wk after surgery. They were housed under controlled conditions of lighting (lights on from 7:00 AM to 7:00 PM) and temperature (22 ± 2°C), with free access to standard rat chow pellets and tap water containing 0.9% (wt/vol) NaCl. Experiments were initiated after a 7-day adaptation period, and hypophysectomized animals gaining more than 5 g during that week were discarded from the study.

Hormones. Methionyl bGH (M1 L126) kindly supplied by Monsanto (St. Louis, MO) was used for injecting the rats and for the development of monoclonal antibodies. The sodium salt of l-thyroxine (T4) and cortisol hemisuccinate (Solu-cortef) were purchased from Aldrich (Milwaukee, WI) and Upjohn (Kalamazoo, MI), respectively. Human IGF-I (International, Amersham, Buckinghamshire, UK) served as tracer and standard in the IGF-I RIA. The bGH used as tracer in the binding studies was a generous gift from Dr. A. C. Paladini (Buenos Aires, Argentina), whereas bGH (B1; AFP-5200) prepared by Dr. A. F. Parlow and obtained through the U.S. Department of Agriculture Animal Hormone Program (National Institutes of Health, Bethesda, MD) was used as unlabeled hormone for the determination of nonspecific bind-
ing. Hormones were labeled with $^{125}$I, with the lactoperoxidase method (16).

Monoclonal antibodies. As described previously (20), anti-bGH MABs were raised by classical methods [culture and cloning of hybridoma cell lines (mouse spleen cells × BALB/C myeloma cells)] secreting mouse monoclonal IGF antibodies against bGH; generation of ascitic fluid by intraperitoneal injection in mice; sodium sulfate purification) and were selected according to their ability to bind $^{125}$I-labeled bGH in solution. Their affinity was determined by Scatchard analysis of displacement of binding (16), obtained with increasing amounts of unlabeled bGH to compete with $^{125}$I-labeled bGH for binding to MABs. One MAB (6B1) that had the most potentiating effects on GH activity was further characterized and selected for subsequent studies (20). A monoclonal antibody directed against a viral protein of bovine leukemia virus (gp 51) was used as a negative control. No potentiating effect of GH action was observed with this MAB anti-gp 51 (data not shown).

Experimental procedure. Hypophysectomized rats were pretreated during 7 days with a daily subcutaneous injection of T$_1$ (1 µg/100 g body wt) and cortisol (50 µg/100 g body wt), which caused body weight to increase slightly in most animals ($\pm$ 5.4 $\pm$ 1.9 g over 1 wk; means $\pm$ SD). Hypophysectomized rats (n = 56) were injected on day 8 with bGH (100 µg/100 g body wt) alone or complexed with anti-bGH MAB 6B1 (40 µg/100 g; molar ratio of MAB to GH = 0.05). Hormone-antibody complexes were allowed to form before injection by preincubation for 3 h at 4°C.

Animals (n = 4/group) were weighed and killed by decapitation without anesthesia 0, 1, 3, 6, 12, and 24 h after a single injection of bGH alone or MAB-bGH complexes. Trunk blood was collected in glass tubes, and the serum was frozen at $-20^\circ$C for determination of IGF-I concentrations. Livers were quickly removed, and a piece was flash-frozen in liquid nitrogen for determination of liver IGF-I mRNA. The remaining tissue was homogenized in 10% (wt/vol) ice-cold sucrose buffer (0.3 mol/l), as described previously (16). The homogenates were stored at $-20^\circ$C until assayed for total and free bGH-binding sites.

Extraction of serum IGF-I and RIA. Serum concentrations of IGF-I were measured by RIA after acid-extraction of serum samples with 0.5 mol HCl/l and silicic acid columns (C$_18$ Sep-Pak cartridges; Waters Associates, Milford, MA), as described previously (17). With the use of this technique, 77 $\pm$ 5% (means $\pm$ SD; n = 4) of the IGF-I was recovered from adult rat serum and $>$99% of the binding protein was removed (17, 23). The human IGF-I antiserum (UB 286) was a kind gift from Dr. L. E. Underwood (Chapel Hill, NC). The sensitivity of this assay was 25 pmol/l, and intra- and interassay coefficients of variation including the extraction procedure were 6.0 and 6.2%, respectively.

Northern blot analysis. RNA was isolated from liver tissue of each animal with the guanidine thiocyanate-cesium chloride method (6). Total RNA was size-fractionated on 1% agarose gels and transferred to nylon membranes (Hybond-N, Amersham, Buckinghamshire, UK) by overnight vacuum blotting (VacuGene XL, Pharmacia LKB, Sweden). After ultraviolet cross-linking (Stratalinker, Stratagene, La Jolla, CA), the membranes were sequentially hybridized to rat $^{32}$P-labeled complementary RNA probes specific for GHR, IGF-I, and IGFBP-3 mRNA, with previously reported prehybridization, hybridization, and washing conditions (30). The specific IGF-I antisense probe was generated from the 194-bp Ava II-Hinf I cDNA rat IGF-I exon 3 fragment inserted into the Bluescript plasmid vector; the GHR/GH-binding protein (GHB) antisense probe was generated from the 951-bp Bgl II rat GHR cDNA fragment subcloned into the pT$_7$T$_3$ vector; and the IGFBP-3 riboprobe was generated from the 452-bp Hind III-BamHI rat IGFBP-3 cDNA fragment inserted into the Bluescript vector, with T$_7$ or T$_3$ RNA polymerase (Life Technologies, Merebeke, Belgium). An 18S $^{32}$P-labeled oligonucleotide was used as a control probe. Relative expression of mRNA in each liver was quantified by scanning autoradiograms with a LKB Ultroscan XL Laser Densitometer (Pharmacia, Sweden). Results were normalized by assigning a value of 1 arbitrary densitometric unit (ADU) to hepatic mRNA from hypophysectomized rats killed at time 0.

Binding studies. Somatogenic binding sites were measured in each rat individually on 2,000 g pellets of liver homogenates previously treated with 4 mol/l MgCl$_2$ to remove endogenous ligand to provide a measure of the total GH-binding sites (18, 19). Parallel studies were performed on water-treated homogenates to measure the free binding sites. Fractional occupancy of the receptors was calculated by the equation: 100 $\times$ (total binding sites − free binding sites)/total binding sites. Specific binding (B) was the difference between the radioactivity bound in the absence and in the presence of unlabeled hormone and expressed as a percentage of the total radioactivity (T): B/T $\times$ 100. MgCl$_2$ treatment caused a protein loss of 19.0 $\pm$ 3.7% (means $\pm$ SD), and the binding values were corrected for this protein loss (19).

Western ligand blot analysis. To assess IGFBPs in serum, ligand blotting was performed as previously described (14). Sodium dodecyl sulfate-polyacrylamide gels (12.5%) were cast with a Hoefer Mighty Small II slab gel system (Hoefer, San Francisco, CA). Electrophoresed proteins were transferred to nitrocellulose membranes that were incubated for 24 h with 250,000 counts·min$^{-1}$·mol$^{-1}$ of $^{125}$I-labeled IGF-II (250 µCi/µg) and were washed and subjected to autoradiography. The autoradiograms were scanned with an Ultroscan XL (LKB) apparatus. For each sample, density signals were determined for the 45,000–39,000 relative molecular weight (Mr) (glycosylated variants of IGFBP-3), 34,000–29,000 Mr (glycosylated variants of IGFBP-1 and -2, and glycosylated IGFBP-4, -5, and -6), and 45,000–39,000 Mr (nonglycosylated IGFBP-4) bands. The 34,000–29,000 Mr (nonglycosylated IGFBP-4) bands. The 34,000–29,000 Mr (nonglycosylated IGFBP-3) bands. The 34,000–29,000 Mr (nonglycosylated IGFBP-4) bands. The 34,000–29,000 Mr (nonglycosylated IGFBP-3) bands. The 34,000–29,000 Mr (nonglycosylated IGFBP-4) bands.

Statistical analyses. Data were analyzed with the BMDP New System professional (University of California Press, Berkeley, 1995) statistical software. All parameters were compared between GH and MAB-GH groups at the different time points with a two-way analysis of variance with Fisher’s F tests when variances were equal, or with Brown-Forsythe F tests when the assumption of equal variances was not satisfied (5). Serum IGF-I, liver IGFBP-3 mRNA, serum IGFBPs, and GH-receptor occupancy values were log transformed before analysis because of a proportional relationship between means and SD in raw data (11). Results are reported as means $\pm$ SE. The P values were corrected for multiple comparisons with a Bonferroni criterion (11). All tests were two-sided, and a P value <0.05 was considered significant.

RESULTS

Injection of MAB-GH complexes in hypophysectomized animals caused both a potentiation and a prolongation of the body weight gain response to bGH. A significant weight gain was already observed 12 h after injection of MAB-GH complexes (2.6 $\pm$ 0.7 g), whereas no change in body weight was recorded after bGH alone at this time ($-0.5$ $\pm$ 0.6 g; P < 0.01). After 24 and 48 h, the body weight gain of the MAB-GH injected rats was

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1.5- and 6-fold higher than that of the rats injected with bGH alone (body wt gain: 6.6 ± 0.7 vs. 4.3 ± 0.5 g; P < 0.05 and 5.9 ± 0.6 vs. 0.9 ± 1.0 g; P < 0.001, respectively).

To further explore the mechanisms of MAb growth-promoting effects, we studied the time-dependent effects of the MAb on the serum IGF-I response to GH. After a single injection of bGH, serum IGF-I concentrations peaked at 12 h (219 ± 37 ng/ml) and rapidly returned to preinjection values by 24 h (Fig. 1). When bGH was injected complexed to the anti-bGH MAb, a higher IGF-I peak was reached after 24 h (295 ± 24 ng/ml; P < 0.01), and after 48 h, serum IGF-I levels were still significantly elevated compared with values observed after bGH alone. When bGH was complexed with a monoclonal antibody directed against an unrelated viral protein (MAb anti-gp 51), no enhancing

Fig. 1. Serum insulin-like growth factor I (IGF-I) concentrations of female hypophysectomized rats at different time intervals after a single sc injection of bovine growth hormone (bGH, 100 µg; ●), either alone or complexed with monoclonal antibody (MAb) 6B1 (40 µg; ○). Results are shown as the means ± SE of 4 rats/group. ***P < 0.001 vs. group receiving bGH alone.

Fig. 2. Top: representative Northern blot analysis of liver IGF-I mRNA in hypophysectomized rats at different time intervals after a single sc injection of bGH (100 µg), either alone or complexed with MAb 6B1 (40 µg). Total RNA (20 µg) were electrophoresed in each lane, and Northern blot analysis was performed as indicated in MATERIALS AND METHODS. 18S, control probe. Bottom: relative abundance of total liver IGF-I mRNA in both bGH (open bars)- and MAb-GH (hatched bars)-injected groups at different time intervals. Results are means ± SE of 4 rats/group. *P < 0.05 vs. bGH alone.
effect was observed on body weight gain or serum IGF-I (data not shown). Further studies were therefore not pursued with this antibody.

We next studied the effect of MAb on the time course of GH-induced IGF-I gene expression. Liver IGF-I mRNA levels were markedly induced by GH injection, with a maximal increase by ninefold at 6 and 12 h (Fig. 2). Similar peak levels were reached after injection of MAb-GH complexes, but thereafter IGF-I mRNA abundance remained significantly higher than after bGH alone (by 65% at 24 h, \( P < 0.001 \) and by 64% at 48 h, \( P < 0.05 \)). This sustained increase of IGF-I gene expression was similarly observed for the four IGF-I mRNA size classes of transcripts (7.5–4.5, 1.7–1.2–0.7-kb transcripts).

Because the IGFBPs may modulate IGF-I clearance and actions, we also investigated the effects of the MAb on serum IGFBPs. GH injection to hypophysectomized rats increased serum IGFBP-1 to -4 (Fig. 3). Serum IGFBP-3 levels were increased fivefold (\( P < 0.001 \)), and serum IGFBP-4 levels were increased twofold (\( P < 0.01 \)) 12 h after bGH injection, whereas IGFBP-1 and -2 levels rose twofold at 24 and 48 h (\( P < 0.05 \)). The MAb did not show any significant potentiating effect on the response of IGFBP-1, -2, and -4 to GH. In contrast, the elevation of serum IGFBP-3 levels in response to GH was markedly amplified and prolonged, being 3.6-fold higher (\( P < 0.01 \)) 24 h after the MAb-GH injection compared with bGH injection alone.

GH administration after 12 h caused a twofold increase in IGFBP-3 mRNA levels (\( P < 0.05 \)) whether injected alone or complexed to the MAb (Fig. 4). This response was sustained in the presence of MAb, as IGFBP-3 mRNA levels remained twofold higher (\( P < 0.01 \)) 24 h after MAb-GH injection compared with levels observed after injection of bGH alone.

To explain this MAb-related prolongation of IGF-I and IGFBP-3 synthesis, we evaluated the effects of the MAb at the hepatic GH-receptor level. We determined its effects on liver GHR and GHBP mRNA levels and on liver GH binding at different time points. After GH injection, a slight increase in liver GHR and GHBP mRNAs levels was observed 12 h after injection of bGH (\( P < 0.01 \) and \( P < 0.05 \), respectively, compared with time 0) (Fig. 5), but addition of the MAb did not modify this slight induction of GHR and GHBP gene expression. On the other hand, total liver GH receptors were not significantly affected by injection of bGH alone or complexed with the MAb (respectively 4.0 ± 0.4 vs. 4.0 ± 0.9 B/T mg protein at 12 h).

After a single injection of bGH or MAb-GH complexes, free liver GH-binding sites decreased rapidly to a similar extent in both groups (−94% after bGH and −95% after MAb-GH at 1 h). In the group treated with bGH alone, free binding sites returned to preinjection values by 12 h, whereas a slower recovery of the free binding sites occurred after MAb-GH injection (4.6 ± 0.5 after bGH vs. 2.4 ± 0.3 B/T × 100/mg protein after MAb-GH, at 12 h; \( P < 0.05 \)). At 24 and 48 h, the number of free binding sites was again similar in both groups. From the determination of total and free binding sites, occupancy of liver GH receptors could be calculated (Fig. 6). This occupancy was nearly complete 1 h after bGH was injected either alone or complexed to the MAb. Thereafter, GH-receptor occupancy decreased rapidly in GH-injected hypophysectomized animals but remained significantly elevated up to 12 h after the injection of MAb-GH complexes, suggesting a sustained binding of GH to its liver receptors when injected complexed to the monoclonal antibody.

**DISCUSSION**

The present time-course study demonstrates that a MAb against bGH both amplifies and prolongs the
serum IGF-I response to GH in hypophysectomized rats. This effect seems to result in part from a prolongation of liver IGF-I synthesis and also from an enhanced induction of IGFBP-3 in serum, leading to an extended half-life of IGF-I in the circulation. Increased liver IGF-I and IGFBP-3 synthesis may in turn be the consequences of sustained binding of GH to its liver receptors in the presence of MAb, as evidenced by our time-course study of GH-receptor occupancy.

We show here that the potentiation of IGF-I response by the MAb is due to amplified and sustained IGF-I levels in the circulation rather than early greater induction of the growth factor. In fact, only the late phase of the GH response is affected by the MAb. Indeed, serum IGF-I levels raised similarly during the first 12 h regardless of the administration of the MAb. Later, the MAb complexed to GH delayed and amplified the peak of serum IGF-I concentrations. MAbs also seem to slow down the return of serum IGF-I to preinjection values. This accumulation of IGF-I in serum might result either from a reduction of the peptide clearance rate from the circulation or from a prolonged synthesis in the presence of the MAb. Our data suggest that both mechanisms might be operative.

No change in body weight was observed 12 h after GH injection, whereas, at the same time, MAb-GH injection already caused a significant increase in body weight. This early effect of MAb on weight gain contrasts with its delayed effects on IGF-I, therefore suggesting that part of the GH effects on body weight is independent of IGF-I stimulation. MAbs could indeed enhance the direct effects of GH on sodium and water retention by the kidney, which are commonly recorded during the initial phase of GH treatment (24). In
addition, MAbs are able to potentiate other anabolic, galactopoietic, and diabetogenic effects of GH unrelated to IGF-I production (1, 25).

GH administration increased serum IGFBP-3 levels, and this induction was markedly potentiated by coadministration of the MAb as higher serum IGFBP-3 levels were observed at 12, 24, and 48 h. This might account in part for the prolongation of high IGF-I concentrations in serum as the half-life of IGF-I is extended when associated with the IGFBP-3 in a ternary complex (32). Evidence for potentiation of IGF-I action by IGFBP-3 is also provided by several studies (7, 9, 10, 13). In particular, it has been shown that injection of human IGFBP-3 together with IGF-I to GH-deficient rats causes significant increase in body weight gain and epiphyseal width compared with IGF-I alone (7). These effects may be related to prevention of type I IGF-receptor downregulation (8) or to the ability of IGFBP-3 to associate with cellular membrane, lowering its affinity for IGF-I and favoring receptor availability of IGF-I (22). Finally, binding protein-complexed IGF-I may constitute a slow-release form that may also temporarily protect IGF-I from degradation until it is released from the complexes by proteolytic processing of the IGFBP, which reduces its affinity for the ligand (34). The marked potentiating effect of MAb-GH coadministration on IGFBP-3 levels may therefore be one of the pivotal mechanisms by which monoclonal antibodies amplify GH growth-promoting actions.

We also show in the present report that the MAb is able to prolong the GH-induced IGFBP-3 mRNA synthesis up to 24 h after injection. The parallel time course between serum IGFBP-3 peptide and hepatic IGFBP-3 mRNA concentrations in response to MAb-GH injection suggests that the MAb potentiates the GH-induced IGFBP-3 levels at the level of gene expression.

In a previous study, we reported that GH-receptor occupancy was increased sixfold 18 h after injection of MAb-GH complexes (20). We now demonstrate that this increase is due to a prolongation rather than an amplification of receptor occupancy and that GHR gene expression and total liver GH-binding sites are not modified in the presence of the MAb. Two separate mechanisms may explain the prolongation of GH binding to its liver receptors: prolonged half-life of the

**Fig. 5.** Top panel: representative Northern blot analysis of liver GH receptor (GHR) and GH-binding protein (GHBP) mRNA in hypophysectomized rats at different time intervals after a single sc injection of bGH (100 µg), either alone or complexed with MAb 6B1 (40 µg). Total RNA (20 µg) were electrophoresed in each lane, and Northern blot analysis was performed as indicated in MATERIALS AND METHODS. Bottom: relative abundance of total liver GHR and GHBP mRNA in both bGH (open bars) and MAb-GH (hatched bars)-injected groups at different time intervals. Results are means ± SE of 4 rats/group. ▲P < 0.05, ▲▲P < 0.01 vs. time 0.

**Fig. 6.** Occupancy of the liver somatogenic receptors in hypophysectomized rats at different time intervals after a single sc injection of bGH (100 µg), either alone or complexed with MAb 6B1 (40 µg). Total and free liver bGH-binding sites were determined respectively in MgCl2- and water-treated homogenates of hypophysectomized rats and from these values, occupancy of liver somatogenic receptors was calculated in each group as described in MATERIALS AND METHODS. Values are means ± SE of 4 rats/group. *P < 0.05 vs. bGH alone.
hormone in serum or retarded internalization of the GH-GHR complex in the presence of the MAb.

Prolongation of the systemic half-life of exogenous insulin may occur in diabetic patients with insulin autoantibodies and may enhance its biological activity (12). Likewise, the MAb anti-bGH might induce prolonged GH levels in circulation, and this would account for prolonged GH-receptor occupancy (3). Serum GH concentrations and receptor occupancy have indeed been shown to correlate well in hypophysectomized rats (18). However, it is unlikely the sole explanation for MAb-mediated enhancement of GH action. Indeed, continuously raised GH levels rather produce attenuated body growth gain and lower serum IGF-I in hypophysectomized rats when compared with intermittent peaks of hormone levels (18). Furthermore, we found that a continuous infusion of GH for 3 days was less efficient to induce body weight gain and serum IGF-I than daily injections of MAb-GH complexes for the same period (unpublished data).

It has been reported that after MAb-GH complexes have been injected in intact rats, their uptake by the liver is delayed, compared with GH alone (28). Moreover, MAb-GH complexes are recovered to a large extent within sinusoidal cells, whereas the hormone alone is mostly found in hepatocytes (28). These observations support the view that prolonged receptor occupancy might be due to modification in the intracellular processing of the GH-GHR complex in the presence of the MAb. Increased affinity of the receptor for GH could also be implicated through allosteric conformational change of the hormone induced by the MAb (21). Indeed in vitro studies show that MAbs that potentiate GH-induced proliferation of cultured Nb2 cells also enhance the affinity of GH for its receptors (26). This possibility cannot be ruled out in our in vivo experiment as Scatchard analysis was not performed.

Little is known about the quantitative relationship between liver GH binding and biological activity. Our data provide support for a tight and sustained coupling between both phenomena. Prolonged receptor occupancy seems to lead to sustained IGF-I synthesis, in agreement with in vitro data showing that incubation of cultured hepatocytes with GH progressively increased IGF-I mRNA levels up to 24 h (29). Furthermore, in hypophysectomized rats, continuous infusion of GH induces a dose-related receptor occupancy that correlates with serum IGF-I levels (18).

In summary, our kinetics study provides new insights into the mechanisms by which monoclonal antibodies may potentiate growth hormone action. The MAbs affect mainly the late phase of the somatogenic response to GH. They prolong the binding of GH to its liver receptors, leading thereby to both a prolonged liver IGF-I synthesis and an increase of IGFBP-3 production. These two mechanisms act concurrently to maintain elevated IGF-I levels in serum and to potentiate GH growth-promoting actions.

We wish to thank P. Malvaux and M. Maes for continuous support. This work was supported by a grant (2.4555.92) from the National Fund for Scientific Research (Belgium).

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Received 13 November 1998; accepted in final form 22 April 1999.

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