Enhancement of growth hormone activity in vivo by monoclonal antibodies: potential for autoimmunization

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Enhancement of hormonal activity by monoclonal antibodies (MAbs) or polyclonal antisera has been observed in a number of systems both in vitro and in vivo. The short biological half-life of many hormones or mediators is, in itself, suggestive that prolongation of activity in the circulation would enhance activity. Indeed, during the course of insulin therapy in diabetes the development of antibodies against the hormone is often associated with an improvement in hormone activity ("depot effect") which, in some cases, appears to be beneficial to the patient. Similarly, in patients who develop antibodies whilst receiving human growth hormone (hGH) therapy, only a small proportion (5%) suffer a decreased growth rate (Ammann, 1986). Antibody-mediated enhancement of insulin activity in vitro has been achieved by virtue of the bivalent nature of antibody molecules (Schecter *et al.* 1979*a*), indicating that the same hormone may have its activity enhanced by distinct mechanisms. A similar antibody-mediated enhancement effect was also observed for epidermal growth-factor activity in vitro (Schecter *et al.* 1979*b*). In both cases the enhancement effect was attributed to facilitated cross-linking of the corresponding receptor through the hormone molecule, implying that the specificity of the antiserum employed must be such that hormone-receptor interaction is permitted.

Enhancement of hGH and bovine growth hormone (bGH) activity in vivo (as measured by increased growth, cartilage metabolism, and muscle protein synthesis) has been demonstrated using hormone–MAb complexes (Holder *et al.* 1985; Aston *et al.* 1986, 1987). The efficacy of the effect appears to depend on the specificity of the MAb in question; however, to date no MAb used alone has inhibited the growth-promoting activity of growth hormone (GH) in vivo. This effect is independent of MAb bivalency or antibody Fc-region-mediated targeting to the liver (Aston *et al.* 1986, 1987).

Wool-growth responses in ewes treated with ox GH were increased in animals showing antibodies to the hormone; it was proposed that decreased hormone degradation was responsible for this increased activity (Ferguson, 1954). Furthermore, improvements in GH potency in vivo have also been noted when GH is administered in a prolonged or continuous fashion, rather than in a single treatment (Clark *et al.* 1985).

In addition to enhancement of the somatogenic activity of hGH and bGH, MAbs have also been shown to enhance the lactogenic actions of hGH and human chorionic somatomammotrophin (hCS). We describe here the effects of anti-hGH and bGH MAbs as well as the binding of polyclonal antisera directed towards the carboxyl-terminal region of hGH on growth rate. It is shown that there is no clear correlation between the effects of different MAbs on hormone-receptor binding and the growth-enhancement

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phenomenon. However, enhancement of bGH activity by certain MAbs is associated with substantial increases in circulating insulin-like growth factor I (IGF-I) levels.

Materials and methods

Hormones and antibodies. bGH was purified as described previously (Wallis & Dixon, 1966) whereas hGH was chromatographically purified from outdated MRC clinical-grade material. IGF-I analogue for iodination was obtained from Amersham International plc, Amersham, Bucks and the standard IGF-I preparation (30 units/mg) was derived from Cohn Fraction IV as described previously (Svoboda *et al.* 1980; Ray & Wallis, 1985). Radioiodination of GH was performed by the lactoperoxidase method resulting in a tracer activity of 6–8 $\times$ 10^7 counts/min per μg (Aston *et al.* 1985). Labelling of IGF-I was achieved by the iodogen method resulting in a specific activity of 270 μCi/μg (Fraker & Speck, 1978).

The MAbs used were prepared by standard techniques (Ivanyi & Davis, 1980; Aston *et al.* 1986, 1987) and have been characterized extensively for their specificity and isotope (Aston & Ivanyi, 1985; Aston *et al.* 1986, 1987). Their effects on hGH and bGH activities in vivo have been examined in Snell dwarf mice (Holder *et al.* 1985; Aston *et al.* 1986, 1987) whereas the effects of MAbs on the lactogenic activity of hGH have been examined in the pigeon crop-sac test (Aston *et al.* 1986). Antibodies to the COOH-terminal fragment (7kDa) of hGH were raised in Balb/c mice by immunization with Freund's complete adjutant. The preparation of this fragment from hGH has been described previously (Aston & Ivanyi, 1983). Antiserum to IGF-I (549/804) was raised in rabbits and was provided as a gift from NIADDK (London).

Hormone treatment of rats Growth rate in hypophysectomized male Wistar rats (Charles River, 125 g) was determined daily after injection of bGH (50 μg) alone or in

Table 1. *Effects of monoclonal antibodies (MAbs) on growth hormone activity in dwarf mice*

Hormone*	MAb†	Affinity (l/nmol)‡	Specificity	$^{35}SO_4^{2-}$ uptake (dpm/mg) Mean	SE	Relative growth Rate (%)§
hGH	EB1	0·43	hGH/hCS	1333	172	592
hGH	EB2	1·00	hGH/hCS	1296	123	576
hGH	QA68	1·00	hGH	223	19	99
hGH	NA71	5·20	hGH	690	57	306
bGH	OA11	0·43	bGH	1195	104	217
bGH	OA12	0·27	bGH	1054	119	191
bGH	OA13	0·31	bGH	1390	47	252
bGH	OA14	NE	bGH	535	42	97
bGH	OA15	0·72	bGH/pGH/rGH	876	70	159
bGH	OA16	0·10	bGH	870	53	158
bGH	OA17	0·36	bGH	601	93	109

NE, not examined; dpm, disintegrations /min; hGH, human growth hormone; bGH, bovine growth hormone; pGH, porcine growth hormone; rGH, rat growth hormone; hCS, human chorionic somatomammotrophin.

* hGH and bGH were administered to six animals, at doses of 10 and 50 μg respectively, after complexing with MAb.

† MAbs were employed at doses of 100 μg (anti-hGH) and 4 μg (anti-bGH).

‡ Determined by Scatchard analysis.

§ Determined by $^{35}SO_4^{2-}$ incorporation into costal cartilage; values for hGH and bGH in the presence of control globulin were 225 (SE 23) and 550 (SE 51) dpm/mg respectively.

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complex with MAb OA11, OA16 or OA17. Complexes between MAb and hormone were prepared 1 h before injection interscapularly (0·5 ml/injection) and blood samples were derived from tail bleeds.

Radioimmunoassay of IGF-I in rat serum. Determination of IGF-I was performed on serum from treated rats after extraction using Sep-paks. Essentially, serum (20 μl) was acidified (0·5 M-hydrochloric acid, 1·0 ml) and applied to a C_{18} Sep-pak cartridge (Millipore, Bedford, Ma, USA) that had previously been washed with 5 ml propan-2-ol, 5 ml methanol and 15 ml acetic acid (40 ml/l). Following sample application, the column was washed with 20 ml acetic acid (40 ml/l) and IGF-I was subsequently eluted with 4 ml methanol. Following evaporation of the methanol, the residue was dissolved in 0·5 ml radioimmunoassay buffer (sodium dihydrogen phosphate 11·6 mM, bovine serum albumin 2·5 g/l, sodium azide 2 g/l, EDTA 10 mM, Tween 20 0·5 g/l, adjusted to pH 7·5 with sodium hydroxide) before radioimmunoassay. Extracts were diluted two- to fourfold and 0·1 ml sample was mixed with 0·1 ml ^{125}I-labelled IGF-I and 0·1 ml anti-IGF-I serum (diluted 1:2500, v/v) and incubated for 16 h at 20°. Antibody complexes were precipitated by centrifugation (3000 g, 20 min, 4°), 30 min after the addition of a solution (0·9 ml) containing (g/l) 167 polyethylene glycol 6000, 0·67 bovine gamma-globulin, 0·5 Tween 20 in 0·05 M-NaH_2PO_4 adjusted to pH 7·6 with NaOH. Results were expressed as ng equivalents IGF-I in relation to the laboratory standard.

Assay of growth in dwarf mice. Use of Snell dwarf mice for the assessment of somatic growth by weight gain and metabolic $^{35}SO_4^{2-}$ incorporation into costal cartilage has been described previously (Holder *et al.* 1980). This strain of mouse is characterized by hypoplasia of the anterior pituitary which results in GH deficiency and small body size. The results presented correspond to means with their standard errors from groups of six animals matched by age and weight.

Fat and protein contents in dwarf mice. The minced carcass of each mouse was analysed for body water, fat and protein content. Water content was determined by freeze-drying the minced carcass to constant weight. Fat content was measured using a modification of the method of Southgate (1971). The dried minced carcass was transferred to a 100 ml

Table 2. *Effects of monoclonal antibodies (MAbs) on ^{125}I-labelled human growth hormone (hGH) and ^{125}I-labelled bovine growth hormone (bGH) binding to liver microsomes and lymphoid cell receptors*

(Doses of MAb employed in radioreceptor assay were as follows: liver microsomes 10^4 ng, NB2 1·0 ng and IM9 100 ng for anti-hGH MAb, whereas for anti-bGH MAb 1·0 μg MAb was employed for microsomes)

	Specific binding in the presence of MAb (%)					
	Liver microsomes				Lymphoid cells	
MAb	Mouse	Rat	Rabbit	Sheep	IM9	NB2
EB1	56	98	79	44	11	40
EB2	59	100	80	18	12	6
QA68	15	30	47	0	82	100
NA71	22	50	25	0	40	46
OA11	—	—	102	300	—	—
OA12	—	—	65	130	—	—
OA13	—	—	69	95	—	—
OA14	—	—	36	100	—	—
OA15	—	—	120	320	—	—
OA16	—	—	27	23	—	—
OA17	—	—	92	250	—	—

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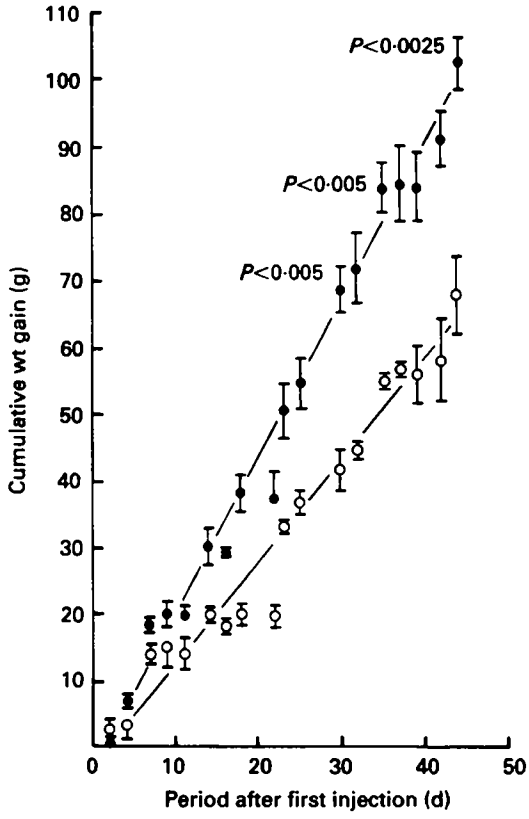

Fig. 1. Enhancement of marmoset growth by passive immunization with anti-human growth hormone monoclonal antibody (MAb)-EB1. Each group (four animals) received treatment three times weekly during the experimental period. (●——●), Animals receiving MAb-EB1 (500 μg/injection); (○——○), animals receiving control immunoglobulin. Points are means with their standard errors represented by vertical bars. Mean values for MAb-EB1-treated animals were significantly different from controls where indicated.

conical flask, 25 ml chloroform–methanol (2:1, v/v) were added, the mixture brought to the boil on a hot plate and filtered using Whatman 541 filter paper; this procedure was repeated three times and the combined filtrates, containing the fat, were dried down in a water-bath. The fat residue was dissolved in 25 ml light petroleum (60–80°), a 10 ml portion was transferred to a weighed container, evaporated to dryness and reweighed. From these values the total fat content was calculated; results were expressed as fat content/animal (g) or as g/kg body-weight.

Protein was calculated from total body-nitrogen content as N × 6·25. N content of the carcass residue was measured by the method of Kjeldahl using a Kjeltec 1300 auto-analyser (Tecator Ltd, Bristol).

Marmoset growth. Juvenile marmosets aged 5–10 weeks (90–140 g) were injected with MAb-EB1 alone or with control mouse globulin. This antibody was selected to study the effects of passive immunization with enhancing MAb since it was the only anti-hGH MAb to cross-react effectively with marmoset GH. Animals were injected with a total volume of 0·5 ml subcutaneously for a period of 6–7 weeks and body-weight was

recorded at injection times. Results indicate the growth rates of groups of four animals (means with their standard errors).

Binding of ^{125}I-labelled GH to receptors/NB2 assay. Microsomal membrane fractions were prepared from liver tissue as described previously (Tsushima & Friesen, 1973) and employed for binding studies as described by Thomas *et al.* (1987). Binding of ^{125}I-labelled bGH or ^{125}I-labelled hGH to microsome receptors, in the presence of various doses of different MAbs, was expressed as a percentage of the total specific binding, which was measured by displacement of tracer in the presence of excess (10^4) unlabelled hormone. Determination of ^{125}I-labelled hGH binding to IM9 cells and the effects of MAbs on NB2 cell proliferation has been described previously (Aston & Ivanyi, 1983; Ivanyi, 1982).

Results

The effects of hGH and bGH, in complex with different MAbs, on the incorporation of $^{35}SO_4^{2-}$ into dwarf-mouse costal cartilage in vivo are shown in Table 1. The correlation between $^{35}SO_4^{2-}$ incorporation and growth rate has been described previously (Holder *et al.* 1985; Aston *et al.* 1987). Although these antibodies represent at least four non-overlapping antigenic epitopes on the corresponding hormones, in no case was inhibition of GH activity observed. The growth rate of complex-treated relative to GH-treated animals varied from no effect to almost a sixfold increase in sulphation activity. In contrast to their effects in dwarf mice, the panel of anti-hGH MAbs inhibited, to varying degrees, binding in in vitro radioreceptor assays (Table 2). This inhibitory

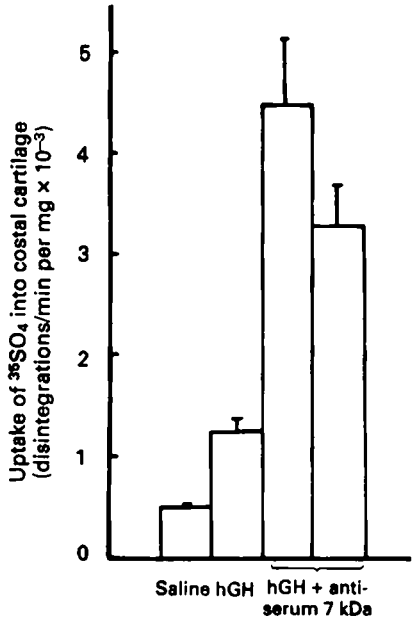

Fig. 2. Enhancement of human growth hormone (hGH) activity by polyclonal antiserum directed to the carboxyl-terminal end of the hGH molecule (7 kDa fragment). Enhancement is shown by two anti-7 kDa antisera. Saline, 9 g sodium chloride/l. For details of procedures, see p. 393. Values are means with their standard errors represented by vertical bars.

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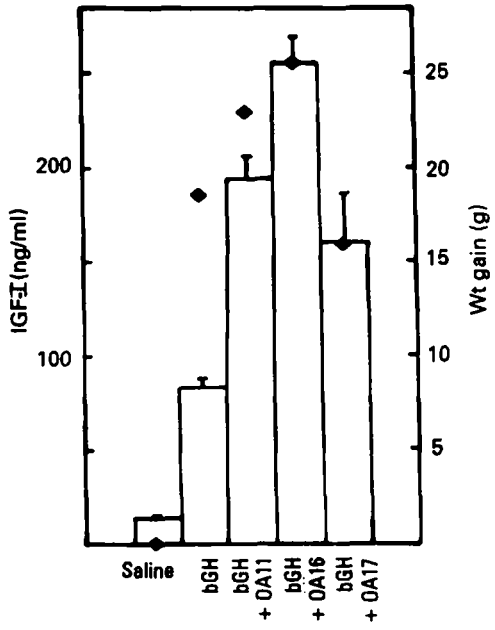

Fig. 3. Potentiation of bovine growth hormone (bGH) activity (weight gain and insulin-like growth factor I (IGF-I) levels in serum) in hypophysectomized rats by anti-bGH monoclonal antibodies (OA11, OA16 and OA17). Saline, 9 g sodium chloride/l. For details of procedures, see p. 393. Values are means with their standard errors represented by vertical bars.

effect of individual MAbs varied, depending on the source of the receptor preparation. Both EB1 and EB2 had little effect on the binding of ^{125}I-labelled hGH to rat liver microsomes but were effective inhibitors of hGH-receptor binding to IM9 cells or sheep liver microsomes. Enhancement of hormone-receptor binding was only observed with

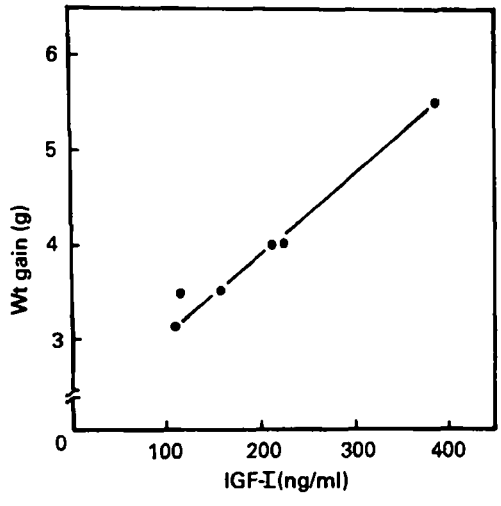

Fig. 4. Correlation between enhanced growth rate and insulin-like growth factor I (IGF-I) levels in hypophysectomized rats treated with bovine growth hormone in complex with monoclonal antibodies OA11. For details of procedures, see p. 393.

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Table 3. *Fat and protein contents, expressed as g/kg body-weight and protein:fat ratio in dwarf mice treated for 10 d with phosphate-buffered saline (9 g sodium chloride/l; PBS controls), human growth hormone (40 mU/d; hGH) or hGH (40 mU/d) with monoclonal antibody (MAb)-EB1 (15000 ABT_{50}/d; hGH/MAb-EB1)*

(Mice received 75% of an *ad lib.* diet. Values are means with their standard errors for six mice/group)

Treatment group . . .	PBS		hGH		hGH/MAb-EB1	
	Mean	SE	Mean	SE	Mean	SE
Fat (g/kg body-wt)	185	7	128***	4	74***†††	6
Protein (g/kg body-wt)	144	2	146	2	165***†††	3
Protein:fat	0·78	0·04	1·15***	0·06	2·34***†††	0·23

Mean values were significantly different from PBS control group (Student's *t* test): ***$P<0·001$.
Mean values were significantly different from hGH alone group (Student's *t* test): †††$P<0·001$.

bGH MAbs; however, their efficacy in this assay did not correlate with their growth-enhancing activities in vivo. This effect was apparent with OA16, which was enhancing in dwarf mice but inhibiting in both rabbit- and sheep-liver microsome-binding assays.

Potentiation of endogenous GH activity was achieved by passive immunization of marmosets with MAb-EB1 (Fig. 1). This antibody was employed as it was shown to cross-react effectively with the phylogenetically-related marmoset GH. Animals receiving EB1, rather than normal mouse globulin, grew significantly more (30–40 %) after 40 d of treatment.

Polyclonal antisera raised against the COOH-terminal, subtilisin-derived fragment of hGH (Aston & Ivanyi, 1983) were found to bind to intact hGH and enhance its activity in dwarf mice (Fig. 2). Due to the substantial loss of antigenicity of this fragment (Li & Graf, 1974; Aston & Ivanyi, 1985) the repertoire of specificities in the anti-7 kDa polyclonal antiserum may be limited.

Enhancement of the growth rate in hypophysectomized rats, by complexing bGH with MAbs, results in significant increases in circulating IGF-I levels after 36 h. Although a good correlation between weight gain and enhanced IGF-I levels was observed for some MAbs this was not apparent in all cases (Figs. 3 and 4; OA11).

In addition to increasing growth rate and serum IGF-I levels, enhancement of GH activity with MAbs has been shown to decrease fat content significantly, increase protein content and increase the efficiency of food utilization (Table 3). These effects are believed to be the result of repartitioning nutrient deposition in favour of accretion of protein rather than fat (Holder *et al.* 1988). Similar effects were observed for various dietary levels (values not shown).

Discussion

Enhancement of GH activity following complexing with MAbs has been demonstrated in several growth models. The extent of the enhancement varies according to the specificity of the MAb employed; however, no clear-cut correlation could be demonstrated between the receptor-inhibitory (or enhancing) activity of the different MAbs and the phenomenon. The observation that certain enhancing MAbs are potent inhibitors of GH binding to some receptor types, but not others, is suggestive of a 'restriction' mechanism operating, i.e. MAbs which enhance growth permit hormone binding to only those receptors involved in the growth process. Such a hypothesis is

supported by the fact that several GH receptor structures and specificities have been demonstrated (Thomas *et al.* 1987; Barnard *et al.* 1985).

Previous studies on the time-course of the enhancement effect (Holder *et al.* 1985) and the increase in lactogenic activity of hGH after binding to MAb (Aston *et al.* 1986) suggests that although the systemic clearance rate of the hormone may be altered, this does not contribute significantly to the enhancement effect. It is still possible, however, that MAbs may prevent degradation of GH in the tissues or alter, in some way, the nature or duration of its interaction with its receptor.

The enhanced growth-promoting activity of GH, following complexing with MAb, is associated with marked increases in circulating IGF-I levels. For certain MAbs the enhancement of growth rate correlates well with the increased IGF-I levels whereas, with others, this is not the case. These observations may indicate that more than one mechanism is involved in the enhancement phenomenon. The fact that site-directed antisera to the COOH-subtilisin-derived fragment of hGH (7 kDa) enhance the growth-promoting activity of the intact hormone also implies that the phenomenon is not dependent on the use of a MAb with unique specificity. Similarly the previous demonstration that univalent Fab' antibody fragments are also effective in this phenomenon excludes bivalency or Fc-mediated targeting as the basis of the effect (Aston *et al.* 1986, 1987). Although this report is concerned with enhancement of GH action, this phenomenon appears to be a more fundamental property of hormone–MAb complexes since we have recently reported the enhancement of thyroid stimulating hormone action (Holder *et al.* 1987). These observations may provide the basis for increasing animal production by immunologically regulating endogenous hormone activity.

The authors would like to thank Julie Blows for expert assistance in the preparation of this manuscript.

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Printed in Great Britain