Binding specificity of monoclonal antibodies towards fragments of human growth hormone produced by plasmin digestion

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To help define the immunological epitopes on human growth hormone (hGH), interaction of fragments of the hormone with 7 monoclonal antibodies (McAbs) was studied. Plasmin-digested hGH, containing two peptides (hGH^{1-134} and $hGH^{141-191}$) joined by a disulphide bond, bound to each McAb with affinity similar to that of intact hGH. The purified C-terminal fragment, $hGH^{141-191}$, showed low affinity for each McAb. The N-terminal fragment, hGH^{1-134}, bound with quite high affinity to 2 McAbs (EB1 and EB3) but not to the other 5. We conclude that residues 1–134 of hGH contain the epitope to which McAbs EB1 and EB3 bind.

Human growth hormone *Monoclonal antibody* *Plasmin digestion* *Radioimmunoassay* *Immunological epitope*

1. INTRODUCTION

Monoclonal antibodies (McAbs) to human growth hormone (hGH) have been described in [1–3]. Each such antibody may interact with just one epitope (determinant) on the monomeric hormone, but different McAbs may interact with different epitopes [1,4–6]. The potential uses of McAbs as probes of structure and function make it important to define the sites with which they interact in as much detail as possible. In order to help define the epitopes on hGH we have modified the hormone by digestion with the enzyme plasmin, and have studied the ability of the fragments so-produced to bind to a series of McAbs.

Plasmin cleaves hGH (a protein with a single polypeptide chain of 191 residues) after residues 134 and 140, liberating a hexapeptide and converting the single chain into a two-chain molecule linked by a disulphide bridge [7]. Two fragments, hGH^{1-134} and $hGH^{141-191}$, can be separated by gel filtration after reduction and alkylation of disulphide bridges. Interaction of the cleaved hGH and the separated peptides with 4 McAbs to hGH, 3 McAbs to human placental lactogen (hPL) (which show almost complete cross-reaction with hGH [6,8]) and with a polyclonal antiserum to hGH has been studied.

Abbreviations: hGH, human growth hormone; McAb, monoclonal antibody; hPL, human placental lactogen

2. MATERIALS AND METHODS

2.1. *Antibodies to hGH*

Antibodies from 4 hybridoma cell lines (NA27, NA39, NA71 and QA68) secreting mouse McAbs (IgG) against hGH and from 3 hybridoma cell lines (EB1, EB2 and EB3) secreting IgG McAbs against hPL were generously provided by Dr J. Ivanyi (Department of Experimental Immunobiology, Wellcome Research Laboratories, Beckenham, Kent BR3 3BS). Their preparation and characterization has been described in [1,6,8]. The three antibodies raised against hPL all show almost complete cross-reaction with hGH [6,8].

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A polyclonal antibody against hGH (RD 15/16) was obtained from Wellcome Diagnostics Ltd.

2.2. *Iodination and radioimmunoassay of hGH*

hGH (prepared by the method as in [9]; a generous gift of Wellcome Laboratories and the DHSS/MRC Joint Advisory Committee) was iodinated with $Na^{125}I$ as in [4], using a modified chloramine T method. ^{125}I-Labelled hGH was purified by gel filtration [4]. It had spec. act. 40–80 μCi/μg.

The immunological activity of hGH fragments was tested using a liquid phase radioimmunoassay system, as in [4], with each of the 8 different antibodies studied. All samples, standards etc. were dissolved in assay buffer containing 0.05 M sodium phosphate (pH 7.6), 0.6 mM merthiolate and either 0.05% bovine serum albumin or 0.05% Tween 20.

2.3. *Plasmin digestion of hGH and preparation and characterization of fragments*

The procedure used was based on that in [7]. hGH (5 mg/ml) was dissolved in 60 mM NH_4HCO_3–NH_3 buffer (pH 8.0) and digested with porcine plasmin (0.2 mg/ml) [from Sigma (London) Ltd.] at 37°C. After 3.25 h the mixture was removed and freeze-dried, after addition of the protease inhibitor Trasylol (100 KIU/ml) (Bayer Ltd., Haywards Heath). For reduction and alkylation, 15 mg plasmin-digested hGH was dissolved in 2.0 ml 8 M urea [in 0.1 M Tris–HCl buffer (pH 8.3)], dithiothreitol was added to 7.5 mM (a 10-fold molar excess over cystine residues), and the solution was flushed with nitrogen for about 5 min and left for 1 h at 20°C. Iodoacetamide (recrystallized from water) was added to give 75 mM. After 1 h at 20°C, the reaction mixture was fractionated directly on a column (93.7 cm × 0.78 cm^2) of Sephadex G-50 fine, equilibrated and eluted at 4°C with 10% (v/v) acetic acid.

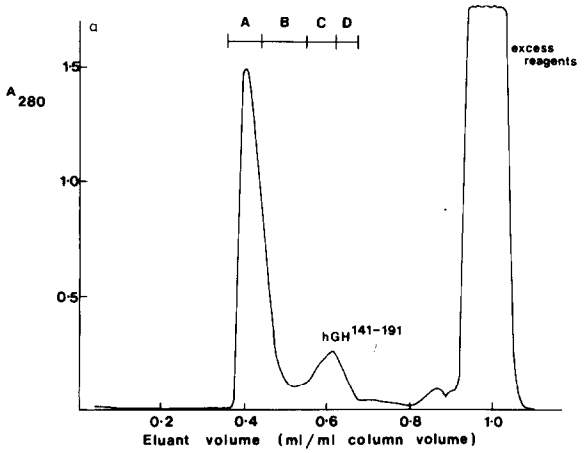
The larger fragment produced by plasmin digestion was rechromatographed on a column (89.2 cm × 0.78 cm^2) of Sephadex G-100 superfine, equilibrated and eluted with 10% (v/v) acetic acid at 4°C. This column was calibrated for M_r-determination using bovine serum albumin, ovalbumin and myoglobin as standard proteins of known M_r.

Polyacrylamide gel electrophoresis in SDS was carried out in slab gels using the discontinuous system in [10]. Amino acid analyses were carried out after hydrolysis with 6 N HCl containing a trace of phenol (110°C, 24 h, sealed evacuated tube), on a Locarte amino acid analyser.

3. RESULTS

3.1. *Digestion of hGH with plasmin*

SDS–polyacrylamide gel electrophoresis of hGH after digestion with plasmin showed the expected pattern (fig.1b, track 7). Two main bands were visible, corresponding to components of M_r ~14300 and <10000, as would be predicted from



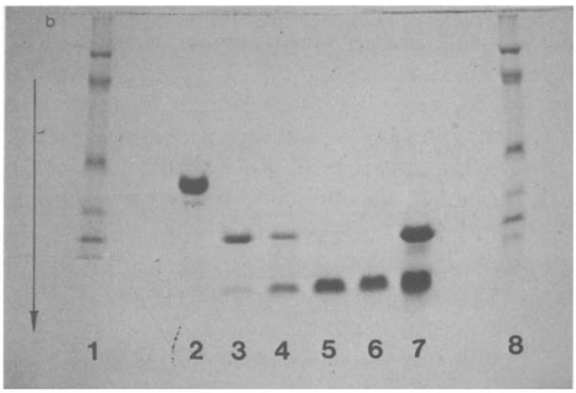


Fig.1. (a) Fractionation of plasmin digested hGH, after reduction and carbamidomethylation, by gel filtration on Sephadex G-50, in 10% acetic acid; (b) polyacrylamide gel electrophoresis in SDS of (1) and (8) markers, (2) undigested hGH, (3) fraction A from the column, (4) fraction B, (5) fraction C, (6) fraction D, (7) plasmin digest of hGH.

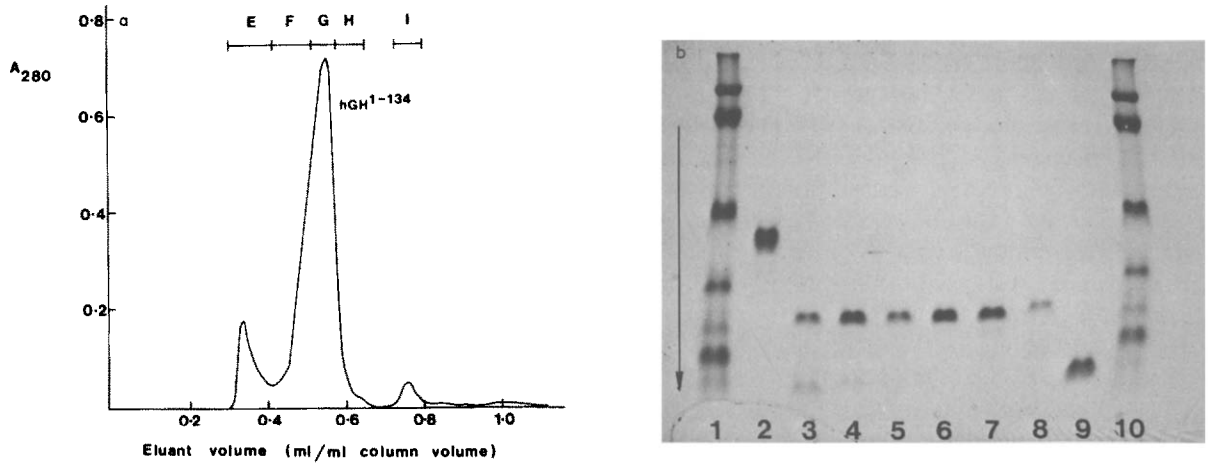


Fig.2. (a) Fractionation of fraction A from the Sephadex G-50 column (fig.1) by gel filtration on Sephadex G-100 in 10% acetic acid; (b) polyacrylamide gel electrophoresis in SDS of (1) and (10) markers, (2) undigested hGH, (3) plasmin digest of hGH, (4) fraction A from fig.1, (5) fraction E from the G-100 column, (6) fraction F, (7) fraction G, (8) fraction H, (9) fraction I.

the results in [7]. Plasmin-digested hGH was reduced, carbamidomethylated and submitted to gel filtration (fig.1a). Two main peptide-containing peaks were detected which corresponded, as demonstrated by SDS–gel electrophoresis, to the two major components seen in plasmin digests (fig.1b, tracks 3–6). However, the first peak includes not only the large plasmin fragment (hGH^{1-134}) but some of the smaller fragment ($hGH^{141-191}$), possibly because of continued non-covalent association between the two on gel filtration. Rechromatography of this component on Sephadex G-100 allowed the isolation of the main plasmin fragment (hGH^{1-134}) (fig.2a) apparently free of the C-terminal component (fig.2b).

The amino acid compositions of the two plasmin fragments corresponded closely to those predicted for hGH^{1-134} and $hGH^{141-191}$.

3.2. *Immunological activity of fragments produced by plasmin digestion of hGH*

The ability of the plasmin fragments to compete with ^{125}I-labelled hGH for binding to each of the 8 antibodies available was studied (table 1). The

Table 1

Relative immunopotency of fragments produced by plasmin digestion of human growth hormone

Antibody	Immunopotency[a]		
	Unfractionated plasmin digest	hGH^{1-134}	$hGH^{141-191}$
NA 27	2.16 ± 0.18	0.014 ± 0.002	0.0062 ± 0.0027
NA 39	2.06 ± 0.12	0.013 ± 0.0005	0.0082 ± 0.0007
QA 68	1.11 ± 0.06	0.011 ± 0.002	0.0063 ± 0.0007
NA 71	1.28 ± 0.05	0.0023 ± 0.0003	0.0047 ± 0.0001
EB 1	1.11 ± 0.06	0.46 ± 0.08	<0.01
EB 2	1.70 ± 0.24	0.024 ± 0.004	<0.01
EB 3	1.49 ± 0.03	0.55 ± 0.04	<0.02
Polyclonal antibody	1.17 ± 0.07	0.0017 ± 0.0004	0.0036 ± 0.0005

[a] Relative to intact hGH

unfractionated plasmin digest possessed an affinity for each of the monoclonal antibodies that was at least as great as that of the unmodified hormone.

With every antibody tested, the affinity of the C-terminal fragment of hGH ($hGH^{141-191}$) was low, in all cases <2% that of unmodified hGH (table 1). With 6 of the 8 antibodies tested the affinity of hGH^{1-134} for antibody was also low, but with 2, EB1 and EB3, substantial interaction between antibody and peptide was observed. When the assay buffer used contained the detergent Tween 20 rather than BSA the immunopotency of all the fragments of hGH (relative to intact hGH) was decreased, but the ratio of the affinity with EB1 and EB3 to that with the other antibodies was increased.

4. DISCUSSION

A McAb to a monomeric protein antigen is expected to bind to just one of several possible epitopes (antigenic determinants). Modification of the antigen by chemical or enzymic means would be expected to alter such epitopes selectively and hence facilitate definition of the epitopes on an antigen such as hGH.

Modification of hGH with plasmin has been studied in detail [7]. Our plasmin digest of hGH contained virtually no unmodified hGH (detectable by polyacrylamide electrophoresis); cleavage on the C-terminal side of residues 134 and 140 appears to have occurred as expected, yielding a two-chain molecule containing hGH^{1-134} linked to $hGH^{141-191}$ by a disulphide bridge. It is likely that the 6-residue peptide $hGH^{135-140}$ separates from the two-chain molecule after digestion. The disulphide-linked plasmin-digested hGH retained an affinity for each of the McAbs studied at least as great as that for unmodified hGH, suggesting that the region between residues 134 and 141 in hGH may not be involved in the determinants for any of these antibodies. A polyclonal antibody against hGH also showed high affinity for the plasmin digested hGH, in agreement with previous reports [11].

When hGH was reduced, alkylated and fractionated by gel filtration, peptides corresponding to hGH^{1-134} and $hGH^{141-191}$ (with reduced and carbamidomethylated ½-cystine residues) were obtained. The first chromatographic separation (Sephadex G-50) gave apparently pure $hGH^{141-191}$, but the N-terminal fragment (hGH^{1-134}) was still contaminated with $hGH^{141-191}$, presumably because of incomplete dissociation of the two. This material retained relatively high immunopotency with all the McAbs (37–44% with EB1 and EB3; 6.6–8.6% with the others, relative to hGH). A second chromatographic step produced hGH^{1-134} apparently free of the C-terminal fragment (though a trace contamination cannot be ruled out).

$hGH^{141-191}$ bound very poorly to all the antibodies tested. In most cases a residual binding, equivalent to a potency of 0.3–1% in the radioimmunoassay, was detected, but whether this reflects an intrinsic activity of $hGH^{141-191}$ or contamination with intact hGH or other fragments cannot be determined at this stage. hGH^{1-134} also showed low potency (0.2–2.5%) in the radioimmunoassay using most of the antibodies, and again the low residual activity could reflect a low intrinsic potency or contamination: Synthetic peptides representing hGH^{19-128}, hGH^{73-128} and hGH^{98-128} have been shown to bind with low affinity (<1% that of hGH) to 4 other McAbs [12]. However, with two McAbs, EB1 and EB3, hGH^{1-134} retained a relatively high binding ability. EB1 and EB3 have been shown to bind to a common epitope [6]; presumably this is largely retained in hGH^{1-134} whereas the epitopes to which the other antibodies bind are not. Low binding affinity could be due to absence of part of the amino acid sequence comprising the binding site or inability of the amino acid sequences present to fold to give the appropriate conformation for the determinant. Affinity of hGH^{1-134} for the polyclonal antibody used was rather lower than reported for antibodies raised in guinea pigs (20% that of hGH [11] or 1–3% that of hGH [13–14]), which could be due to differences in the antibodies or in the handling of the peptides. It is possible that alkylation of the ½-cystine residue in hGH^{1-134} (and/or the 3 ½-cystines in $hGH^{141-191}$) contributes to the low binding ability of the fragments for some antibodies, but this seems unlikely since reduced and alkylated (but uncleaved) hGH retains high binding affinity for all of the antibodies (unpublished).

Different results have been obtained using fragments obtained by subtilisin digestion of hGH

and the same McAbs as those used here [15]. The larger (15 kDa) fragment, probably hGH^{1-139} (possibly mixed with some larger peptides) retained ability to bind to all of the McAbs tested, though binding to NA71 was relatively weak. In addition, EB1 and EB3 also bound to the smaller subtilisin fragment (7 kDa) corresponding approximately to $hGH^{140-191}$. The differences between our results and those in [15] may be due to differences in the detailed structures of the fragments studied, but they could also be due partly to the use of different methods for detection of immunoactivity.

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REFERENCES

[1] Ivanyi, J. and Davies, P. (1980) Mol. Immunol. 17, 287–290.
[2] Bundesen, P.G., Drake, R.G., Kelly, K., Worsley, I.G., Friesen, H.G. and Sehon, A.H. (1980) J. Clin. Endocrinol. Metab. 51, 1472–1474.
[3] Retegui, L.A., DeMeyts, P. and Masson, P.L. (1982) Prot. Biol. Fluids 29, 827–832.
[4] Wallis, M., Surowy, T.K. and Ivanyi, J. (1982) Mol. Cell. Endocrinol. 28, 363–372.
[5] Cadman, H.F., Wallis, M. and Ivanyi, J. (1982) FEBS Lett. 137, 149–152.
[6] Ivanyi, J. (1982) in: Monoclonal Hybridoma Antibodies: Techniques and Applications (Hurrell, J.G.R. ed) pp.59–79, CRC Press, Boca Raton FL.
[7] Li, C.H. and Gráf, L. (1974) Proc. Natl. Acad. Sci. USA 71, 1197–1201.
[8] Ivanyi, J. and Davies, P. (1982) Prot. Biol. Fluids 29, 855–860.
[9] Lumley Jones, R., Benker, G., Salacinski, P.R., Lloyd, T.J. and Lowry, P.J. (1979) J. Endocrinol. 82, 77–86.
[10] Laemmli, U.K. (1970) Nature 227, 680–685.
[11] Clarke, W.C., Hayashida, T. and Li, C.H. (1974) Arch. Biochem. Biophys. 164, 571–574.
[12] Retegui, L.A., Milne, R.W., Cambiaso, C.L. and Masson, P.L. (1982) Mol. Immunol. 19, 865–875.
[13] Li, C.H. (1975) Proc. Natl. Acad. Sci. USA 72, 3878–3882.
[14] Mills, J.B., Kostyo, J.L., Reagan, C.R., Wagner, S.A., Moseley, M.H. and Wilhelmi, A.E. (1980) Endocrinology 107, 391–399.
[15] Aston, R. and Ivanyi, J. (1983) EMBO J. 2, 493–497.