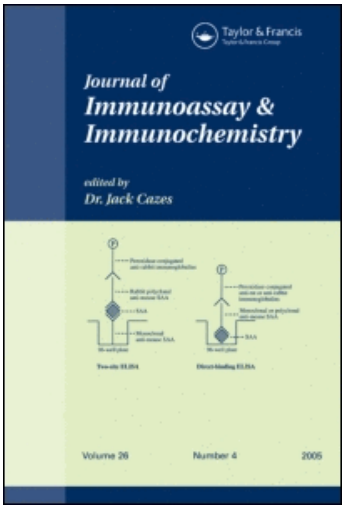

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A HIGHLY SENSITIVE RADIOIMMUNOASSAY FOR HUMAN GROWTH HORMONE USING A MONOCLONAL ANTIBODY

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ABSTRACTS :

Biozzi-strain mice were immunized with a highly purified preparation of 20K variant of hGH. Spleen-cells were fused with SP2/0Ag14 myeloma cells.
Clone productions were screened for specificity toward 20K and 22K hGH and for the affinity constant of antibody-antigen reaction. For the selected monoclonal antibody, Ka was $1.02.10^{11}$ L/M using 22K hGH as both tracer and reference preparation. No cross reactivity was found with PRL and other pituitary hormones ; hPL reactivity was 0.002 percent that of hGH.
According to these antibody characteristics, a highly sensitive RIA system was developed and used for specific GH measurement in human serum. Using logit-log co-ordinates, the slope of the standard curve was -1.099 and the minimum detected dose was 0.5 uIU/ml.
Excellent correlation (r = 0.9575) was found between assay data in this system and those of a conventional RIA method using specific polyclonal rabbit antiserum.
The International Reference preparation (66/217) could adequately be used to calibrate the monoclonal antibody system since the in house internal 22K GH standard and international one were equally well recognized by the monoclonal antibody.

(KEY WORDS : Human Growth Hormone - Monoclonal Antibody - Radioimmunoassay).

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INTRODUCTION

HGH and other polypeptide hormones are routinely measured by radioimmunoassay (RIA) using polyclonal antibodies but these antisera often cross react with structurally related molecules of the same family.
Indeed, 85 % of the amino acid sequence residues of hGH is homologous with human placental lactogen (hPL) and 16 % with human prolactin (hPRL)(1).
Somatic cell fusion techniques generate large series of clones secreting antibodies of different specificity and affinity, each of them directed against one epitope of the antigen. The characteristics of the monoclonal antibody (Mab) remaining constant and their obtention theoretically unlimited, they are ideal reagents for the development of diagnostic techniques providing they fulfill specific requirements.
This work describes the selection of an anti hGH monoclonal antibody suitable for detection of the low levels of the hormone in serum. Its specificity and the affinity constant of its binding reaction with the hormone compare well those of previously selected anti-hGH Mab (2).

MATERIALS AND METHODS

Reagents :
The following preparations were consistently used throught the work : the International hGH Reference Preparation MRC 66/217 (2 U/mg) ; highly purified 22K and 20K forms of hGH, Eq.GH, bGH and pGH (3); the UCB-Bioproducts preparations calibrated against MRC Standards : hPL (1 U/g - MRC standard 75/545), hPRL (34 U/mg -MRC standard 75/504), hCG (9900 U/mg - MRC standard 75/537), hCG alpha (1000 U/mg - MRC standard 75/569), hFSH (1500 U/mg - MRC standard 69/104), hLH (7000 U/mg - MRC standard 68/40), hTSH (4,5 U/mg - MRC standard 68/38).

Immunization :
Three doses (20 ug, 20 ug and 5 ug) of a preparation of the 20K variant of hGH, emulsified in complete Freund's adjuvant, were injected subcutaneously

on day 0, 30 and 120. in "high responders selected" mice of the Biozzi strain (4).

A boosting injection of 10 ug was administered intra-peritoneously (I.P.) on three consecutive days i.e. 5, 4 and 3 days before fusion. The total dose injected was 75 ug per animal.

Cell fusion, hybridoma production and first screening :

Mouse spleen was perfused with RPMI 1640 (Gibco Europe) and 1 X 10^8 spleen cells were fused with 3 X 10^7 SP_2O ag 14 mouse myeloma cells according to the general technique of Köhler and Milstein (5) with some modifications (6,7).

Fused cells were distributed and cultured in microcultures plates in selective HAT-DMEM medium (8) supplemented with 15 % foetal calf serum (FCS).

Harvested media of microcultures plates were tested for GH antibody production by enzyme - linked - immunoadsorbent assay (ELISA). The positive ones were then controlled by RIA.

The tracer and RIA conditions.

Purified hGH (10 ug in 20 ul of 0,2 M borate buffer) were added to 25 ug of Iodogen (1,3,4,6-tetrachloro-3,6-diphenyl glycoluril, Pierce (9) using the method described by Salacinski et al (10) with some modifications.

^{125}Iodine (1 mCi, Amersham IMS 30) was then added, and the mixture incubated for 10 minutes at room temperature.

The iodination mixture was taken up in 0,5 ml sodium phosphate buffer (0.05 M, pH 7.4) and loaded on an Ultrogel (LKB) ACA 54 column (10 mm x 300 mm) equilibrated in the same buffer to separate fully immunoactive hGH tracer from damaged forms and free iodine. The tracer exhibited a specific activity of 80 uCi/ug as calculated by self displacement experiments.

The radioimmunoassay conditions were the following : tracer (50.000 cpm) was added to each tube as 200 ul phosphate buffer containing one percent normal mouse serum. Incubation (500 ul final volume) was performed 15 to 20 hours at room temperature. Separation of bound and free tracer was made by precipitation of the immune complexes by 2 ml per tube of goat antimouse antiserum diluted (1 % V/V) in a 8 % (W/V) of polyethyleneglycol 6000 solution (UCB, Belgium).

Hybridoma selection and antibody characterisation.

A first degree selection was made on the basis of the extent of the tracer binding (^{125}I-GH, 25.000 cpm, 100 ul) given by the antibodies present in the culture supernatant (100 ul). The selected colonies were transferred into 2 ml wells (Costar cluster tray 3524).

A second degree selection was performed after five days of culture on the basis of cross reactivity with hPL. When 0,5 ug of hPL was unable to significantly displace the tracer from the antibody in the RIA conditions, the colonies were selected and cells were frozen and stored in liquid nitrogen.

Ultimate selection was based on the data of the complete study of the following antibody characteristics by RIA : specificity, sensitivity (or minimum detected dose), ED_{50} and affinity constant of the binding reaction. For these studies, displacement curves were established using graded doses of hGH, hPL, hPRL, hCG, hCG alpha, hFSH, hLH, hTSH, EqGH, bGH, pGH. All dilutions were made in phosphate buffer (0.02 M, pH 7.4 + 0.5 % BSA).

RIA data were treated, according to the programs of Faden and Rodbard (11) and Rodbard et al (12), on a PDP 11/23 computer. Statistically validated displacement curves were set and the following parameters were established

The sensitivity as the dose of antigen responsible for a radioactivity count statistically different from that given by the binding of the tracer to the antibody, in the absence of antigen.

The ED50 defined as the amount of cold hormone required for 50 % inhibition of the binding of the tracer in the absence of cold hormone, in the conditions defined for the assay ;

The cross reactivity as given by the ratio :

$$\frac{ED50\ hGH}{ED50\ of\ any\ other\ antigen} \times 100$$

in situation where displacement curves were statistically parallel.

The affinity constant as determined according to Scatchard (13)

Colonies producing antibodies with high affinity and strict specificity were then cloned by the limiting dilution technique and positive monoclonal cell lines were subcloned several times until stability.

The original characteristics and parameters were reassumed for the final selected monoclonal antibody production and monoclonal cell line was then frozen and stored in liquid nitrogen.

Monoclonal antibody standard solution.

In vitro production of monoclonal anti-hGH antibody was performed by amplified culture of the established clone, grown to a maximum density in 30 ml and 250 ml culture flasks. The supernatant were pooled, sodium azide (0.2 % W/V) and Trasylol (200 U/EKI/ml - Bayer) added.

The solutions, as 2 ml aliquots, were freezed dried and stored at 4°C.

Monoclonal antibody were also produced in ascitic fluids by an intraperitoneal (I.P.) injection of 6 to 10 x 10^6 cells into hybrid mice (Balb C X Biozzi's mice) which had been given 0.5 ml Pristane (Aldrich - Europe) ten days before cells injection.

Optimisation of the assay for clinical use.

For clinical use, the standard curves were established with the International Reference Preparation MRC (66/217) diluted in a horse serum giving a serum protein effect similar to that of GH-free human serum. Indeed, in serum the radioactivity bound in the absence of growth hormone differed from that in the presence of buffer only.

- *The serum protein effect was estimated from cumulative experiments and calculated in terms of B/Bo ratio were B was the radioactivity of the precipitated tracer-antibody complex of zero point set in serum and Bo that of its counterpart set in buffer.*
- *The Recovery test was performed by adding graded doses of GH in a human serum of known GH content, aliquots being then submitted to the assay and the correlation line between the measured concentrations and the expected ones was then established.*
- *The Test of parallelism used a GH-rich human serum serially diluted with the above mentioned horse serum. Statistical parallelism was estimated according to the program of Faden and Rodbard (11).*
- *The within assay coefficient of variation (C.V.) and the between assay one were performed and computed according to the "Internal Quality Control for Radioimmunoassay" program proposed by Rodbard (14).*

- Correlation studies were performed by comparing the data of GH measurements in 85 human sera as given by the present assay and by a conventional radioimmunoassay using a specific polyclonal antibody (rabbit anti-hGH serum, Techland, Belgium). Computation was made according to BMDP-1 R and 6 D programs (15).

RESULTS

Immunization :

Before boosting, mice serum samples were tested for antibody production by RIA. Maximum binding of the tracer (MxB) by the serum of the selected mouse was 65 % of the added tracer. Serum dilution for 30 % of ^{125}I-GH binding was 1 X 10^{-4}.

Hybridoma selection :

Fifty percent of growing colonies were found to be positive in the ELISA screening for anti hGH antibody production.

By RIA, 159 culture supernatants showed more than 35 % of the added tracer. This figure dropped to 32, one month later. Only five of them showed minimal cross reactivity with hPL and were selected for complete study by RIA. On the basis of the selection criteria the best one (coded 5B4) was selected and submitted to cloning.

Cell lines were considered established when 100 % of wells occupied by cells, initially distributed by the limiting dilution technique were found positive for anti-hGH antibody production. This required up to five succesive clonings.

Established subclones were repeatedly tested for specificity as further proof of monoclonality. Consistancy of specificity was used as a criterium for selection of the clone consequently used for antibody production.

Antibody characteristics :

The original stock solution of the antibody had to be diluted 1 x 10^4 times to achieve 35 % of ^{125}I-GH binding.

The RIA system developed thereof, exhibited the following characteristics :

- Slope : -1.099 $\pm$ 0.138 (n = 55).

- *Sensitivity : 0.5 uIU/ml.*
- *ED_{50} hGH : 11.4 uIU/ml ± 2.18 (N = 55).*
- *No cross reaction was detected with hPRL, hCG, hCG alpha, hTSH, hFSH, hLH, bGH and pGH.*
- *Positive cross reactions, computed on the basis of parallel line assay, were found for the 20K variant, eGH and hPL as shown in table 1.*
- *The affinity constant, Ka was found to be 1.02 x 10^{11} L/M, as computed from data treatment of the displacement curves.*

Optimisation of the assay for clinical use

As seen in fig. 1 and 2 the requirements of the recovery test and that of parallelism were fulfilled by the Mab assay system. The dilution test permitted to set a line with the assay data obtained from the samples resulting from serial dilution of a hGH rich serum. This line exhibited a significant parallelism with the linearized standard curve. Mathematical analysis of the parallelism was set in table 2.

For the first control serum, the mean value was 9.92 uIU/ml (N = 29), the within assay C.V. was 3.95 % and the between assay C.V. was 9.8 %.
For the second control serum, the mean value was 28.25 uIU/ml (N = 29), the within assay C.V. was 2.81 % and the between assay C.V. was 10.30 %.

Serum protein effect (% B/Bo) was 83.9 % for the Mab system and 83.3 % for the polyclonal one.
The sensitivity was found 1.6 greater in the Mab system than in the conventional polyclonal one.
Data on hGH measurements in 85 human sera representing a large range of GH concentrations were compared using the present method and the conventional RIA system referred above.

As seen in figure 3, correlation between the two set of data gave a regression line responding to the equation y = 0.85646 X + 0.35519 with r = 0.9575 and y representing data from the RIA developed with the polyclonal system.

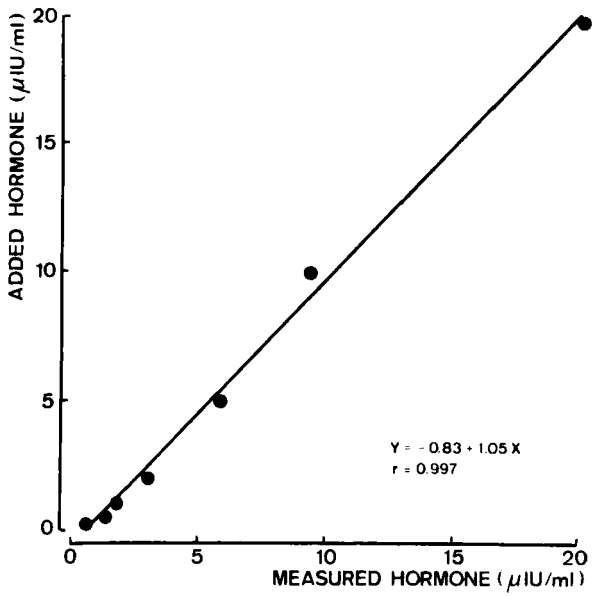

Fig. 1. *Recovery test : the correlation line is plotted with measured hormone concentration as X and known amounts of added hormone as Y.*

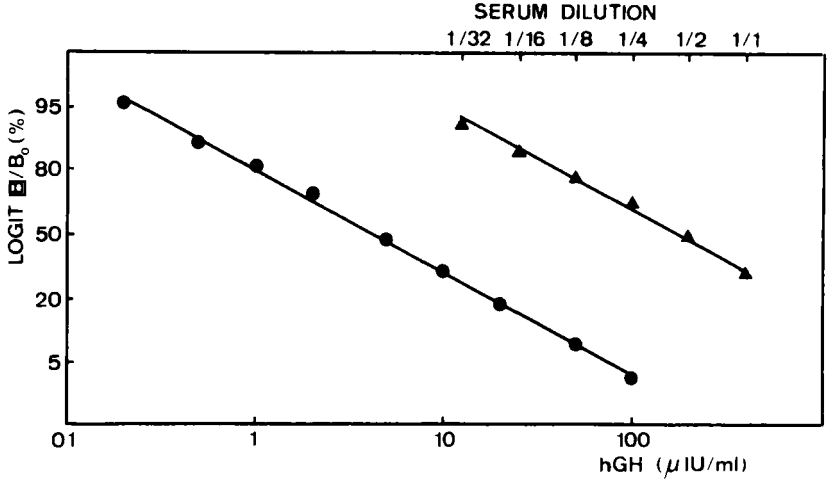

Fig. 2 *Test of parallelism : Comparison of the slope given by several dilutions of a GH rich serum and the International standard (hGH 66/217) assayed using the Mab system.*
No significant difference in slope was found using a t-test.

Table 1

Specificity of the RIA system developed with the monoclonal antibody 5B4.

	% OF CROSS REACTIVITY.
20K variant of hGH	50
Equine - GH	0.0071
hPL	0.002

Table 2

Analysis of parallelism between the standard curve and the serial dilutions of a GH rich human serum.

	Slope	Residual variations	N	r	
Standard curve	-1.023	0.674	12	-0.997	
Serum dilution	-1.032	1.942	12	-0.992	
Potency •	Lower limit •	Upper limit •	% C.V.	F-test +	Test of parallelism ++
20.3	18.7	22.2	3	2.8803 *	0.2164

• in uIU/ml

+ $F_{0.95} = 4.8443$ with $df_1 = 1$ and $df_2 = 11$

++ $t_{0.95} = 2.0860$ with $df = 20$

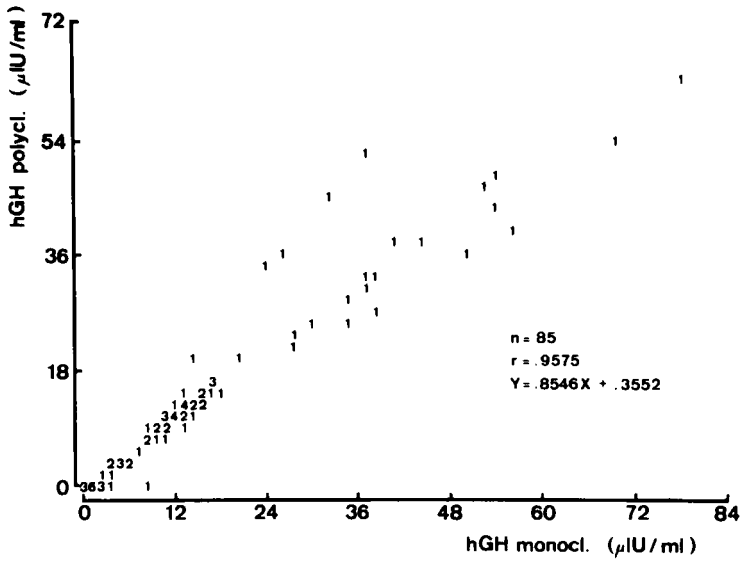

Fig. 3 *Comparison of hGH concentrations in human serum samples measured using the MAb system (abscissa) and using a conventional polyclonal system (ordinate). The individual numbers in the graph represent the number of different sera for which the hGH measurement was found equivalent (e.g. 3 means that three different sera contain the same amount of hGH).*

DISCUSSION AND CONCLUSIONS

In 1980, Ivanji and Davies (16) described eight different hybridoma secreting monoclonal anti-hGH antibodies. Even though no hPRL cross reactivity were registered with two, none of them could be utilized in a diagnostic RIA system due to insufficient affinity of their binding reaction. Bundesen et al (17) produced ten monoclonal antibodies with similar characteristics. Immunochemical studies were further performed by Retegui et al (18), Ivanji (19), Wallis et al (20) and Wallis and Daniels (21) using clones already referred to by Ivanji and Davies (16).

Stuart et al (2) described six high affinity monoclonal antibodies directed against 3 different epitopes of the GH molecule with only one reasonably specific (3 % cross reactivity with hPL) and yielding a Ka of 4,4. 10^{10} L/M.

This brief review stresses well the advantages in raising monoclonal antibodies reacting specifically and with a high affinity for growth hormone. Our work validates such an antibody.

Human growth hormone is present in the pituitary tissues as two dominant entities, variants 22K and 20K. The latter, 10 % by weight in tissue extract, exhibits a specific primary structure where the sequence 32 to 46 is missing. Biologically, the 20K variant possesses most of the actions of its 22K counterpart (3). Since the precise pathophysiologic relevance of 20K GH is still largely unknown, we felt appropriate to develop an immunoassay for it, obtaining first monoclonal anti 20K hGH antibodies with high specificity.

Nevertheless, no stable hybridoma secreting specific anti 20K antibodies could be obtained despite selective immunization of the animals with a highly purified preparation of that variant (3).

Instead, the antibodies reacted systematically better with the 22K hGH and among these, some exhibited an excellent specificity.

This could will be due to the fact that the immunogen, presenting a restricted amino acid sequence as compared to the 22K hGH, possesses epitopes more strictly related to the general GH molecular model.

Consequently a highly specific radioimmunoassay system of required sensitivity and devoid of significant non-specific effect could thus be developed and applied for clinical use. Its characteristics are such that it could be readily introduced as a simple radioimmunoassay method for GH measurements, in serum, avoiding the development of cumbersome and less sensitive immunometric methods. Further, correlation between serum GH measurements in the proposed system and that using conventional polyclonal Ab showed that the assay using the Mab could be easily standardized by a well characterized 22K hGH preparation (3) which appears immunologically identical to the relevant International Standard.

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