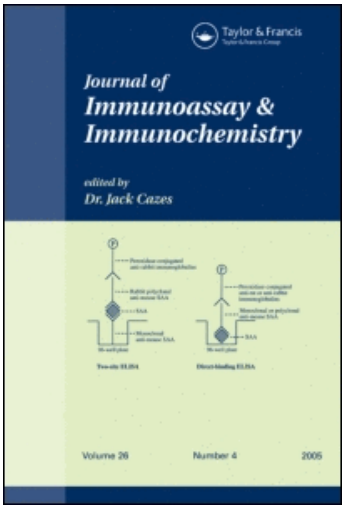

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Identification of Antigenic Differences of Recombinant and Pituitary Bovine Growth Hormone Using Monoclonal Antibodies

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(KEY WORDS: Bovine Growth Hormone, Monoclonal Antibodies, ELISA, Affinity, Crossreactivity)

ABSTRACT

For characterization and determination of recombinant bovine GH (rbGH) eight monoclonal antibodies (MAb) were produced against rbGH from Monsanto. The various MAb showed different affinities to rbGH, pituitary bovine GH (pbGH), and pituitary ovine GH (poGH). With epitope analysis several MAb were shown to recognize different epitopes of rbGH. The MAb MUC-rbGH-3A11 and MUC-rbGH-1E5 were used to develop a Sandwich ELISA. By checking the specificity of the assay no cross reactivity was

found with pituitary porcine GH, pituitary human GH, bovine or ovine prolactin and little cross reactivity with poGH could be found. The Sandwich ELISA detected various rbGH (Monsanto, Elanco, Cyanamid) with different N-terminal amino acids and discriminated between rbGH and pituitary bovine GH by an affinity factor of 2.0. The detection level was 2 ng rbGH per ml PBS buffer. The recovery was about 86% in bovine serum. It might therefore be possible to detect rbGH-treated cows using a Sandwich ELISA, but this would need a field study.

INTRODUCTION

The ability to stimulate body weight gain and to increase milk production in dairy cows by application of crude pituitary extracts is well known. New biotechnical methods have made it possible to produce large amounts of a suitable growth hormone (GH). The application of recombinant bovine growth hormone (rbGH) produced by DNA methodology to increase the output of domestic animals is currently being widely discussed throughout the world. Administration of rbGH has already taken place in some countries, while others request further experiments. Beside studies on eventual adverse effects on animal health, ethical aspects as well as consumer protection have to be considered. An important prerequisite for both a ban on the use of rbGH, as well as for its use, is the ability to reliably detect rbGH in living animals.

The detection of GH has until now been based on radioimmunoassays (1, 2, 3). MAbs allow for the characterization of the antigens' epitope structure. As rbGH and pituitary bovine GH (pbGH) differ in their N-terminal amino acid sequences, epitope discrimination could be expected. Secchi et al. (4) already used an ELISA system for cross-reactivity studies with bGH-specific monoclonal antibodies. In earlier studies Krivi and Rowold (5)

were only able to measure variations in the binding site properties of GH-specific MAb at high GH concentrations.

In this study MAbs against different epitopes of rbGH were produced in order to find antibodies with weak or no cross reactivity to pbGH. The intention was to develop an ELISA sytem with this MAb, which could be routinely used for the detection of bGH and for discrimination between pbGH and rbGH.

MATERIALS AND METHODS

Hormones and chemicals

Different recombinant bovine GH (rbGH) were obtained from Monsanto (LOT M-117-06078), Düsseldorf, FRG, and from Cyanamid (LOT 6776), Briston, USA, from Elanco (LOT 5892), Greenfield, USA. Sequence variations of these various bovine GH (bGH) are shown in table 1. Pituitary bovine GH (pbGH) was obtained from Eli Lilly and Company (LOT A18-7DT-170A), Indianapolis, USA, and from NIH (bGH-NIH-B1), pituitary ovine GH (poGH) from NIH (NIH-GH-S1), pituitary porcine GH (ppGH) from NIH (USDA-pGH.B1), pituitary human GH (phGH, LOT OG1950/3) from Hybritech, Köln, FRG, bovine prolactin (bPRL) from NIH (NIH-P-B3), and ovine prolactin (oPRL) from NIH (NIH-P-S-12).

Bovine serum albumin (BSA), lipopolysaccharide from *salmonella minnesota* RE 595 (LPS), and orthophenylendiamine (OPD) were purchased from Sigma, Deisenhofen, FRG. Complete and incomplete Freund's adjuvant (CFA, IFA) were obtained from DIFCO Laboratories, Detroit, USA, polyethylene glycol 1500 (PEG) and horseradish peroxidase (HRP) from Boehringer, Mannheim, FRG, protein G-sepharose from Pharmacia, Uppsala, Sweden, subtype-

specific goat anti mouse antisera and peroxidase conjugated goat anti-mouse IgG from Medac, Hamburg, FRG, and all other reagents from Merck, Darmstadt, FRG.

Production and isolation of monoclonal antibodies

Female BALB/c mice were immunized with 100 µg rbGH (Monsanto) in 0.1 ml 0.9% NaCl and 0.1 ml CFA s.c. and boosted i.p. 4 weeks later with 100 µg rbGH in 0.1 ml 0.9% NaCl and 0.1 ml IFA. After a further 4 weeks, the mice received 100 µg rbGH in 0.1 ml 0.9% NaCl ip. whereby 10 µg LPS were administered as mitogen (6). On day 5 after the last immunization, hybridization of spleen cells and hybridoma cells (P3-X63-Ag8.653) with PEG followed, using the principle developed by Köhler and Milstein (7), modified as described by Peters et al. (8).

Cell culture supernatants were screened for specific antibody production by a non competitive enzyme immunoassay. Polystyrene ELISA microtiter plates (maxisorp, Nunc, Wiesbaden, FRG) were coated with 2 µg rbGH per ml coating buffer (0.1 mol Na_2CO_3, pH 9.6, 100 µl/well) and left overnight at 4°C. Nonspecific binding sites were subsequently blocked with 1% BSA in PBS, pH 7.2 (200 µl, 1 h at 37°C). Cell culture supernatants (1:2 in PBS-Tween, 50 µl) were applied and incubated for 60 min at 37°C. Specific MAb were detected with goat anti mouse IgG antibodies conjugated to HRP (50 µl/well, 1:500 in PBS-Tween, 1 h at 37°C) and made visible with 100 µl OPD solution (2.5 mmol/l in 0.1 mol/l citrate buffer pH 5.0 with 0.015% H_2O_2). The enzyme reaction was stopped after 10 min with 50 µl 2 mol/l HCl and measured at 492 nm. Between each step, ELISA plates were washed three times with NaCl-Tween (0.5% Tween).

ELISA positive colonies (absorbance > 0.5) were cloned at least twice by the limiting dilution technique (9). Immunoglobulin isotyping was carried

out with immunodiffusion (10) using subtype specific goat anti-mouse antisera. Larger quantities of MAb were produced by spinner and roller cellculture techniques (Tecnomara, Fernwald, FRG). The MAb were isolated from cell culture supernatants with protein G-sepharose (11). Cell culture supernatants or purified MAb were stored at -20°C until use.

Characterisation of the GH preparations

The protein concentrations of various bGH were measured using the Bradford protein assay (12). As standard the purified preparation of rbGH (Monsanto) was used in concentrations between 25 µg/ml and 500 µg/ml. To check the purity of the bGH preparations a SDS-gel-electrophoresis was performed with a 5 - 22.5% gradient gel using the system of Laemmli (13) modified as described by Schranner and Lösch (14).

MAb specificity and affinity determination

The specifiticies and affinities of the obtained MAb were estimated by means of an indirect competitive ELISA (15). ELISA plates were coated with rbGH (2 µg/ml coating buffer) 100 µl per well overnight at 4 °C and blocked with 1% BSA (in PBS pH 7.2, 1 h at 37°C, 200 µl/well). Concentrations of the MAb were set in the non competitive ELISA to get an extinction of about 1.0 (see table 2). The different rbGH's (Monsanto), pbGH (NIH) and poGH (NIH) were pipetted in concentrations of 1 µg/ml or 5 µg/ml, or PBS buffer as control (50 µl), to 50 µl of the MAb. After an incubation period of 1 h at 37°C the binding of the MAb to the plate bound rbGH was detected with goat anti-mouse IgG antibody coupled to HRP (50 µl/well, 1:500 in PBS-Tween, 1 h at 37 C). OPD solution was again applied as substrate.

Epitope analysis

Bovine GH with a molecular weight of 22,000 has been described to have four epitopes (5). The simultaneous antigen binding of different antibody pairs was tested in a Sandwich ELISA using each combination of antibodies. For this purpose all MAb were labelled with HRP using the periodate technique (16). Concentrations were set in the non competitive ELISA to get an extinction of about 1.0.

The ELISA plates were coated with the different MAb overnight at 4°C (2 µg/ml, 100 µl/well) and blocked with 1% BSA in PBS pH 7.2 (1 h at 37°C, 200 µl/well). Then the antigen rbGH was incubated in concentrations of 10 µg, 1 µg, 100 ng and 10 ng per ml PBS-Tween (1 h at 37 °C, 100 µl/well). HRP labelled MAb were applied as second antibodies (1 h at 37°C, 100 µl/well) and made visible with OPD solution.

Development of a Sandwich ELISA for the quantitative assay of rbGH

The MAb MUC-rbGH-3A11 and MUC-rbGH-1E5 were suitable for a Sandwich ELISA because they bind to different epitopes of the bGH (see results).

For optimizing the Sandwich ELISA the plates were coated with various concentrations of MUC-rbGH-3A11 (100 µl/well, overnight at 4 C) and blocked with 1% BSA in PBS pH 7.2 (100 µl/well, 1 h at 37 C). Recombinant bGH (50 ng/ml) was diluted (log_2) in PBS pH 7.2 (50 µl/well) and different incubation times were investigated. As conjugate several concentrations of HRP-conjugated MUC-rbGH-1E5 were used. Again OPD solution served as substrate.

TABLE 1. Sequence variation of various rbGH preparations according to pbGH.

bGH	n-terminal amino acid sequence
pbGH	Phe-Pro-
rbGH (Monsanto)	Met-Phe-Pro-
rbGH (Cyanamid)	Met-Asp-Gln-Phe-Pro-
rbGH (Elanco)	Met-Phe-Pro-Leu-Asp-Asp-Asp-Asp-Asp-Lys-Phe-Pro-

Specificity determination of the Sandwich ELISA

In order to characterize the specificity of the Sandwich ELISA the cross-reactivities with poGH, ppGH, phGH, bPRL and oPRL were studied. Comparison of antibody binding properties of pbGH (NIH, Eli Lilly) and rbGH (Monsanto, Cyanamid, Elanco) which differ in the N-terminal extension (see table 1) was carried out.

Identification of rbGH in bovine serum

With regard to the practical use, it is of interest to detect rbGH in bovine serum. Various concentrations of rbGH (Monsanto, 3 to 50 ng/ml) were in vitro added to a serum pool from 20 cows. Recovery data using a rbGH standard in PBS-Tween were estimated using two modes of incubation (1 h at 37°C or overnight at 4°C).

Statistics

The intra- and interassay variability was determined using a rbGH standard

diluted in PBS-Tween and in bovine serum. The least detectable concentration of rbGH was the concentration resulting in a value higher than two standard deviations from the zero dose response. Statistics were performed with Mann-Whitney test.

RESULTS

Production and characterization of monoclonal antibodies

Four independent fusions resulted in 39 anti-rbGH MAb-producing hybridoma clones. Eight of them were cloned and propagated further. The obtained MAb, isolated by affinity chromatography with protein G sepharose, were named MUC-rbGH-1A1, -1E5, -1E11, -2E10, -3A11, -3G3, -4D6, and -4F5. Six of the MAb were identified as IgG_1 and two as IgG_{2a} (MUC-rbGH-2E10, -4D6). Affinity was determined in indirect competitive ELISA. Depending on the affinity of respective MAb, antibody binding sites were inhibited by adding 1 µg/ml or 5 µg/ml of free rbGH (Monsanto), pbGH (NIH) and poGH (NIH). The rate of inhibition showed considerable differences between the respective antibodies and growth hormons (see table 2). Compared with pbGH (NIH) and poGH (NIH) the MAb MUC-rbGH-3A11, -4F5, -1E11, -3G3 and -1E5 showed a higher affinity to rbGH (Monsanto). The only MAb of this group that had a higher affinity to pbGH than to poGH was MAb MUC-rbGH-1E5. Of all MAb MUC-rbGH-3A11 had the highest affinity to rbGH (Monsanto) with an inhibition of 90.6 %. This MAb also showed a high affintity to the plate coated antigen as an extinction of 1.17 was reached at a concentration of 160 ng/ml. MAb MUC-rbGH-4D6, -2E10 and -1A1 showed an even higher affinity to coated rbGH. However, they could hardly be inhibited by free GH (5 µg/ml). MAb-MUC-rbGH-2E10 and -4D6 had a

TABLE 2. Affinity and specificity testing of the obtained MAb. The affinities to rbGH (Monsanto) coated to microtiterplate were determined in a non-competitive ELISA. The indirect competitive ELISA was carried out with GH concentrations of 1 µg/ml resp. 5 µg/ml. Specificity was tested to rbGH (Monsanto), pbGH (NIH) and poGH (NIH). Results are shown in percent inhibition.

MAb	MAb (in ng/ml)	extinction (492 nm)	GH (in µg/ml)	Inhib. rbGH (Mons)	Inhib. pbGH (NIH)	Inhib. psGH (NIH)
MUC-rbGH-3A11	160	1.17	1	90.6 %	75.3 %	82.9 %
MUC-rbGH-4F5	600	1.15	1	89.0 %	63.8 %	78.4 %
MUC-rbGH-1E11	1000	0.93	1	48.8 %	5.0 %	28.4 %
MUC-rbGH-3G3	1000	1.17	1	34.0 %	10.9 %	27.7 %
MUC-rbGH-1E5	5000	0.95	1	57.9 %	29.2 %	8.7 %
MUC-rbGH-4D6	20	0.83	5	8.9 %	9.1 %	0.0 %
MUC-rbGH-2E10	10	0.56	5	12.0 %	34.8 %	24.5 %
MUC-rbGH-1A1	40	0.95	5	0.0 %	0.0 %	0.0 %

TABLE 3. Epitope fine specificity determined by two site epitope mapping in Sandwich ELISA. The simultaneous binding of MAbs to rbGH (Monsanto) was carried out in each combination. The MAbs were used as coating antibodies and detection antibodies (MAb-HRP). The antigen rbGH (Monsanto) was used in different concentrations (10 ng/ml to 1000 ng/ml).

MAb-HRP	coating MAb							
	-3A11	-1E5	-4F5	-1E11	-3G3	-2E10	-4D6	-1A1
MUC-rbGH-3A11	-	-	+	+	-	-	-	-
MUC-rbGH-1E5	+++	-	+++	+++	+++	-	-	-
MUC-rbGH-4F5	-	-	-	-	-	-	-	-
MUC-rbGH-1E11	-	-	-	-	-	-	-	-
MUC-rbGH-3G3	-	-	-	-	-	-	-	-
MUC-rbGH-2E10	-	-	-	-	-	-	-	-
MUC-rbGH-4D6	-	-	-	-	-	-	-	-
MUC-rbGH-1A1	-	-	-	-	-	-	-	-

extinction (492 nm) > 0.5 using different rbGH (Monsanto) concentrations:
+++ 10 ng/ml ++ 100 ng/ml + 1000 ng/ml - > 1000ng/ml

better affinity to pbGH than rbGH. However their affinity to pbGH was higher than to poGH.

In order to determine the epitopes of bGH recognized by MAb a Sandwich ELISA was carried out. Each coating MAb was combined with every HRP-conjugated detection antibody. As demonstrated in table 3, only the MAb MUC-rbGH-3A11, -4F5, -1E11, and -3G3 in combination with MUC-rbGH-1E5-HRP showed an extinction higher than 0.5 using a rbGH concentration of 10 ng/ml.

Development of a Sandwich ELISA for the quantitative assay of rbGH

After optimization the Sandwich ELISA was carried out as follows: The

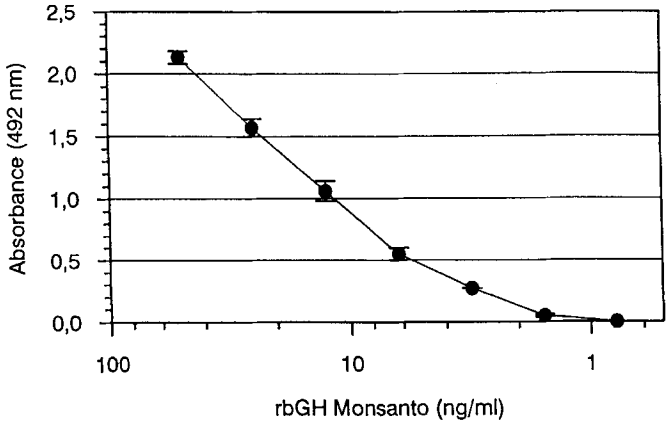

FIGURE 1. Detection of rbGH (Monsanto) using a Sandwich ELISA coating with MUC-rbGH-3A11 and detecting with MUC-rbGH-1E5. The curve shows the absorbance (492 nm) using different quantities of rbGH (Monsanto; n=8, SD interassay).

ELISA plates were coated overnight with 2 µg MAb MUC-rbGH-3A11 per ml coating buffer at 4°C (100 µl/well) and then blocked with 1% BSA in PBS pH 7.2 for 1 h at 37°C (200 µl/well). As a standard rbGH was incubated overnight at 4°C with 50 µl per well in a $\log_2$ dilution series (50 ng rbGH/ml to 0.78 ng rbGH/ml). The second, peroxidase labelled MAb MUC-rbGH-1E5 (diluted 1:300) was applied as conjugate (50 µl/well, 1 h at 37°C). The enzyme reaction was made visible with OPD. Between each incubation step the ELISA plates were washed three times with NaCl-Tween. The titration curve of rbGH (Monsanto) is shown in figure 1.

Specificity determination

In order to check the specificity of the Sandwich ELISA, cross reactivities

towards poGH, ppGH, phGH, bPRL, and oPRL were tested. There was no cross reactivity at all with 50 ng/ml ppGH, 49 ng/ml phGH, 800 ng/ml bPRL, and 800 ng/ml oPRL and some cross reactivity with poGH (31.1 %).

For the development of a specific detection system the antibody binding properties were determined comparing various rbGH samples (Monsanto, Cyanamid, Elanco) and pbGH (NIH, Eli Lilly) in the described Sandwich ELISA. The protein concentrations of GH preparations were measured by Bradford protein assay (Bradford, 1976) to make sure that identical concentrations of the hormones were used. The purity of the various bGH was tested in SDS-gel-electrophoresis (figure 2). All bGH probes proved to be pure and showed a band at a molecular weight of 22,000 with the exception of rbGH from Elanco which had a band at 23,000. The additional bands of pbGH (NIH) and rbGH (Cyanamid) at a molecular weight of 44,000 seemed to be a dimeric bGH.

Recombinant bGH (Monsanto), which was chosen for immunization, was better recognized than pbGH (NIH) and pbGH (Eli Lilly). Compared to rbGH (Monsanto), which was defined as 100 %, the detection rate of all pbGH was significantly lower (43.0 % NIH, 52.8 % Eli Lilly, $p < 0.0001$). In the Sandwich ELISA system rbGH (Cyanamid; 95.5 %) and rbGH (Elanco; 108.2 %) showed nearly the same affinity. Taking these results together the Sandwich ELISA was able to distinguish between rbGH and pbGH by a factor of about 2 (see figure 3).

Quality control of the Sandwich ELISA

To study the practical application of the Sandwich ELISA we examined the rate of recovery of rbGH (Monsanto) in a serum pool of 20 lactating cows. When using an incubation period of 1 h at 37°C for the serum

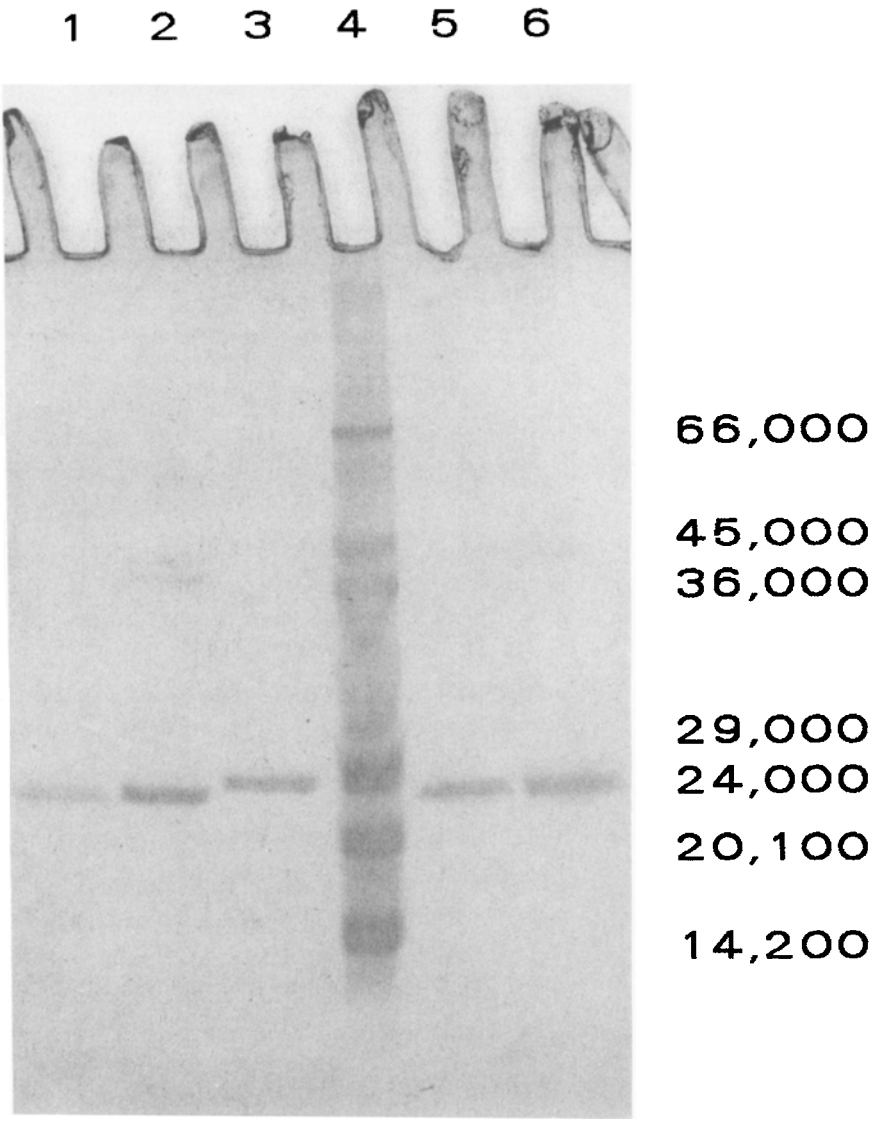

FIGURE 2. SDS gel electrophoresis in a linear pore gradient (5 to 25.5 %). Lane 1, pbGH (Eli Lilly); lane 2, rbGH (Cyanamid); lane 3, rbGH (Elanco); lane 4, marker proteins (7L, Sigma, Deisenhofen, Germany); lane 5, rbGH (Monsanto); lane 6, pbGH (NIH).

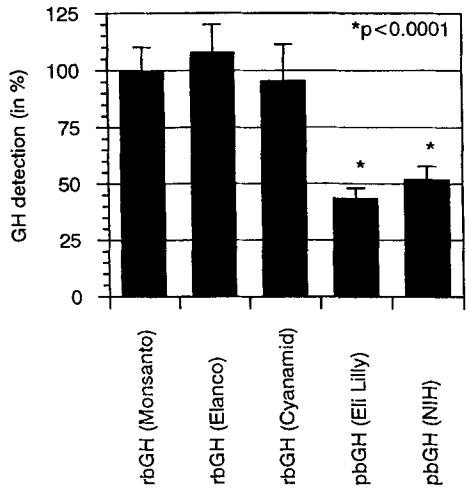

FIGURE 3.Detection of various bGH in Sandwich ELISA. The preparations of rbGH (Monsanto, Elanco, Cyanamid) and pbGH (Eli Lilly, NIH) had identical concentrations (25 ng/ml). The results were shown in percent to rbGH (Monsanto) which was estimated at 100 % (n=6, SD).

probes a recovery rate of 73.0% was found (n=6; SD 7.7 %), whereas an incubation time of 16 h at 4°C led to an increase in recovery to 86.1 % (n=5; SD 4.3 %).

Intraassay reproducibility was < 4.1 % (ICV). Performing the ELISA on eight consecutive days led to an interassay C.V. of < 6.9 %.

Discussion

Numerous GH's have been immunologically characterized since it has become possible to produce MAb. Aston et al. (17) have described four non-overlapping epitopes of the human growth hormone. Krivi and Rowold

(5) reported of similar results for the bovine growth hormone. The advantage of non-overlapping epitopes is the ability to simultaneously bind two antibodies. This makes it possible to detect bGH probes with two specific antibodies when using a sandwich detection system. Since this system is not competitive, the antibodies can be used in higher concentrations.

Krivi and Rowold (5) produced a pbGH specific MAb which was able to distinguish between the pbGH and rbGH. However, a higher affinity of their MAb for pbGH than for rbGH was only observed at concentrations higher than 100 ng/ml. But, if the intention is to detect rbGH in sera of treated cows, very much lower concentrations can be expected.

In this study we described several rbGH specific MAbs. Pairs of them were able to recognize and bind to different epitopes of rbGH at the same time. The best results were obtained when using the MUC-rbGH-1E5 in combination with MUC-rbGH-3A11, -4F5, -1E11 or -3G3. The MAb MUC-rbGH-1E5 could not be used as coating antibody because of a lack of biological activity when bound to polystyrol plates. But this MAb was very suitable as detection antibody.

A competitive indirect ELISA was carried out to test for affinity of the MAb. Monoclonal antibodies MUC-rbGH-3A11, -4F5, -1E11 and -3G3 which performed identically in the epitope studies, showed a higher affinity to rbGH (Monsanto) followed by poGH and pbGH (NIH). The highest affinity to rbGH (Monsanto) was found with MUC-rbGH-3A11 which was then used in Sandwich ELISA.

Monoclonal antibody MUC-rbGH-1E5 showed a different binding pattern, prefering rbGH (Monsanto) and detecting pbGH (NIH) more readily than poGH (NIH). This MAb also recognized an epitope that was different to all the other MAb described previously. The third group of MAb MUC-rbGH-1A1, -4D6 and -2E10 prefered microplate bound bGH with an high affinity. The

MAb MUC-rbGH-1A1 was not even capable of binding 5 µg/ml of free GH. This observed change in the conformation of GH when bound to a solid phase has also been described by Retegui and Paladini (18). The MAb MUC-rbGH-4D6 and -2E10 prefered, in contrast to the other MAb, pbGH in these binding studies. According to the results of the Sandwich ELISA, there might be no difference in binding pattern in indirect competitive ELISA using different rbGH samples. The combination MUC-rbGH-3A11 with MUC-rbGH-1E5 recommended itself in epitope and affinity studies for the Sandwich ELISA.

Up to now, bGH has been detected in samples with the aid of radio-immunoassays (2, 3, 18). In this study we have developed a Sandwich ELISA with a detection limit of 2 ng rbGH per ml which is in a similar range as Krivi and Rowold (2). However, an ELISA has the advantage that the detection of rbGH can be routinely performed in any laboratory. According to Secchi et al. (4) in this ELISA system no cross reactivity to oPRL, bPRL or phGH was found. But in contrast no cross reactivity to ppGH and only little to poGH could be found (4). The rate of recovery was about 86 % in bovine serum. Reproduction testing of this Sandwich ELISA showed satisfactory variabilities of intraassay (< 4.1 %, n=6) and interassay (< 6.9 %, n=8).

Beside the satisfactory detection limit it was very interesting that there were differences in affinity of the MAb to pbGH and rbGH probes by a factor of about 2 in the described Sandwich ELISA. Until now this is not adequate as yet for a forensic identification of rbGH. Nevertheless, our results give reason to hope that in the future a specific detection system for bGH probes of different origins can be found. It can be shown, however, that small differences in structure, for example through additional *N*-terminal amino acids, can markedly change the immunogenic characteristics of a protein.

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