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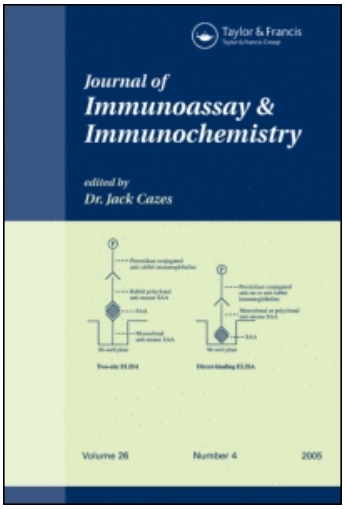

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CHARACTERIZATION OF ANTIBODIES AGAINST HUMAN N-TERMINAL PARATHYROID HORMONE BY EPITOPE MAPPING

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ABSTRACT

Two polyclonal antisera from goat and mouse and two monoclonal antibodies against human parathyroid hormone (1-34) were characterised by epitope mapping. Hexapeptides were synthesized on polystyrene pins, the sequences of which overlapped and represented the entire sequence of hPTH(1-34). Binding of antibodies to these hexapeptides was determined and antigenic determinants thus characterized. At least one predominant binding sequence was detected in the region of hPTH(7-14).
(KEY WORDS: parathyroid hormone, epitope mapping, monoclonal antibodies)

INTRODUCTION

Measurement of peptide hormones in plasma has been much improved by the use of two-site radiometric assays instead of conventional radioimmunoassays. This is especially true for assays of thyrotropin, parathyroid hormone (PTH), adrenocorticotropic hormone, and calcitonin, which have become available in recent years. The development of two-site assays is, however, more

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difficult compared with radioimmunoassays. Usually, one has to screen several antibodies to find a suitable combination. This problem becomes more difficult with small molecules due to the likelyhood of steric hindrance between any two binding antibodies. It is useful therefore to characterize the binding specificity of each antibody.

Classically, binding sites of antibodies against PTH were characterized by using iodine-125 labeled fragments of PTH in binding studies (1) and, additionally, by displacement studies using several different labeled and unlabeled fragments of PTH (2,3,4,5,6). Those studies were laborious and required many different fragments (1-34, 1-12, 13-34 , 18-24, 18-34, 23-34, 25-34, 28-48, 32-43, 44-86, 53-84).

To obtain a more precise characterization and to circumvent the need for many different labeled and unlabeled peptide fragments, we used the epitope mapping technique (7) as a new approach.

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MATERIALS AND METHODS

Reagents

N,N-dimethylformamide (DMF), piperidine, trifluoroacetic acid, triethylamine, phenol, diisopropylethylamine, BSA and OVA were supplied by Serva (Heidelberg, Germany). Dichlormethane, Tween 20, 2-mercaptoethanol and sodiumdodecylsulphate (SDS) were from Sigma (Deisenhofen, Germany) and ethanedithiol from Aldrich (Steinheim, Germany).

Buffers

ELISA buffer (supercocktail) was sodium phosphate buffer(PBS) (66 mM, pH 7.4), containing 1% OVA (w/v), 1% BSA and 0.1% Tween 20. For washing, a solution of PBS/Tween 20 was used (0.9% NaCl, 1% disodium hydrogen orthophosphate, 0.4% sodium dihydrogen orthophosphate, pH 7.2, 0.5% Tween 20). For the enzymatic reaction, citrate-buffer, 0.1 M, pH 7.4 containing 400 µl 30% w/v H_2O_2 and 600 mg OPD (o-phenylenediamine, DAKO, Hamburg, Germany) per liter was used .

Antisera

A polyclonal antiserum against the N-terminal part of PTH was raised by immunizing a goat with extract from human adenomatous parathyroid glands. After repeated booster immunizations, a specific antiserum for hPTH(1-34) was obtained (8).

Monoclonal antibodies were raised in BALB/c-mice. The mice were immunized with synthetic hPTH(1-38) (Bissendorf, Braunschweig, Germany), covalently linked to BSA (9). Each of the antisera was tested for specific binding of hPTH(1-34) by performing a radioimmunoassay. To detect antibodies against hPTH(1-34) specifically, we used the double antibody separation technique and as tracer biotin-hPTH(1-34) labeled with ^{125}I-streptavidin (10). After cell fusion and HAT selection (11),selected hybridomas were screened in the same way. The sera of the mice used for producing hybridomas were tested by epitope mapping and additionally tested for cross reactivity with hPTH(44-68) and hPTH(53-84).

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Epitope Mapping

Epitope mapping was performed by using a commercial kit (CBS, Cambridge, U. K.). Hexapeptides were synthesized on a solid phase (polystyrene pins) covering the complete amino acid sequence of hPTH(1-34) (according to Niall et al., (12)). The hexapeptides were synthesized according to Geysen by linkage of esters of Fmoc-amino-activated-aminoacids. The hexapeptides were synthesized in duplicate and covered hPTH(1-34) in an overlapping way, i. e. first pin: sequence 1 to 6, second pin: sequence 2 to 7, etc.

Detection of binding Antibodies by ELISA

The ELISA was performed in duplicate on microtiterplates (96 wells immunoplates, Nunc, Roskilde, Denmark). Each pin was disrupted and sonicated according to the manufacturers instructions. All incubation and washing steps were performed in microtiterplates during agitation. Pins were precoated with 200 µl of supercocktail per well for 1 h at room temperature. Pins were then incubated with 175 µl of antiserum at 4°C overnight (goat antisera were used diluted 1000-fold in supercocktail, mouse antisera diluted 750-fold, and monoclonal antibodies undiluted as cell culture supernatant). After washing 4 times (10 min) with PBS/Tween 20, the pins were incubated at room temperature with 175 µl of second antibody conjugate for 1 h at room temperature. The washing procedure was then repeated and the pins incubated with substrate solution. The reaction was stopped by removing the pins and adding 50 µl of 1 M HCl to each well. Microtiterplates were read immediately at 492 nm

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in a microplate reader. Values were not corrected for non-specific binding.

RESULTS

The polyclonal goat antiserum raised against native hPTH(1-84) bound 30 % of the tracer peptide hPTH(1-34) at a dilution of 1:1000 (total activity of added tracer per tube: 30,000 cpm). hPTH(44-68) and hPTH(53-84) were bound to less than 0.1 %.

The specifity of the antiserum was determined by epitope mapping and is shown in Fig. 1.

The largest antibody population of this antiserum was directed against the sequence 6-15 (pin 5-10, Gln-Leu-Met-His-Asn-Leu-Gly-Lys-His-Leu). Other epitopes were, however, also recognized, e. g. sequences 16-21 (pin 16, Asn-Ser-Met-Glu-Arg-Val) and 23-28 (pin 23, Trp-Leu-Arg-Lys-Lys-Leu).

The polyclonal mouse antiserum against hPTH(1-38) was more heterogenous than the goat antiserum (Fig. 2). There were at least 4 different epitopes binding significant amounts of antibodies: sequences 5-12 (pin5-7, Ile-Gln-Leu-Met-His-Asn-Leu-Gly), 16-25 (pin 16-20, Asn-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg), 24-30 (pin 24-25, Leu-Arg-Lys-Lys-Leu-Gln-Asp) and 27-34 (pin 27-29, Lys-Leu-Gln-Asp-Val-His-Asn-Phe).

Four supernatants of hybridomas against hPTH(1-38) were also further characterized. All did cross-react with hPTH(44-68) and hPTH(53-84) to less than 0.1 %. Two of them were tested by epitope mapping. The monoclonal antibody A 1-70 did apparently recognize two distinct epitopes (Fig. 3): sequences 12-19 (pin 12-14, Gly-Lys-His-

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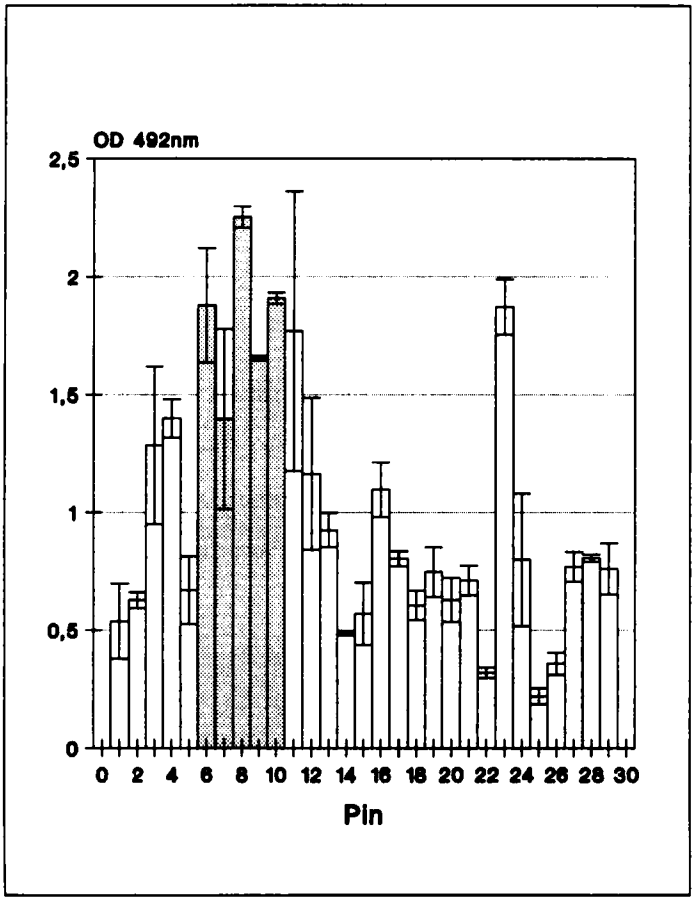

Figure 1. Binding characteristics of a polyclonal goat antiserum against native hPTH(1-84) determined by epitope mapping. y-axis: optical density at 492 nm, maximum value, lowest value, mean value (beam), binding sequences marked. x-axis: hexapeptide on pin 1 (hPTH(1-6)) to hexapeptide on pin 29 (hPTH(29-34)).

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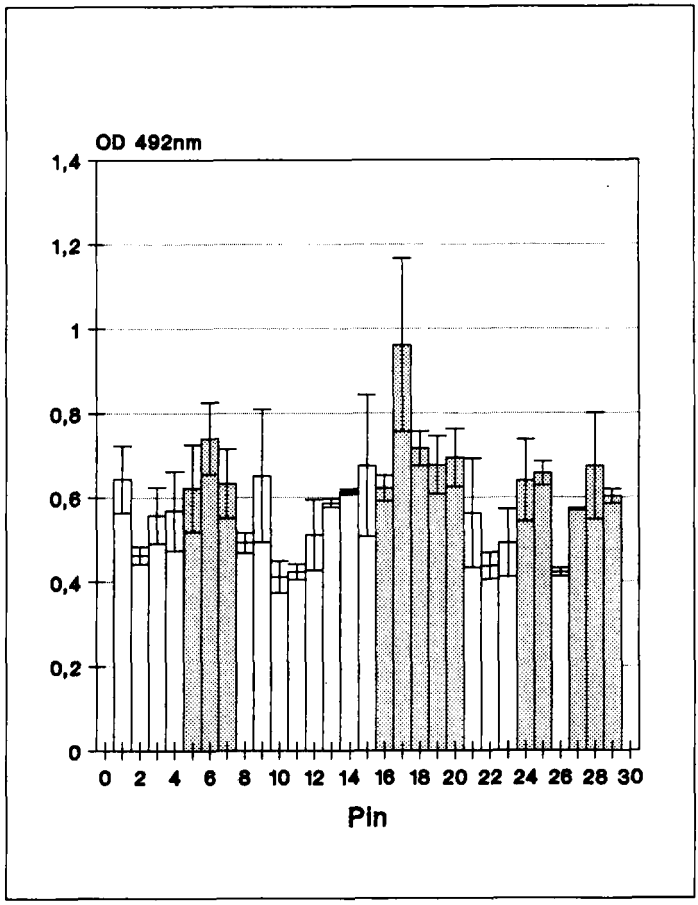

Figure 2. Binding characteristics of a polyclonal mouse antiserum against hPTH(1-38) determined by epitope mapping. y-axis: optical density at 492 nm, maximum value, lowest value, mean value (beam), binding sequences marked. x-axis: hexapeptide on pin 1 (hPTH(1-6)) to hexapeptide on pin 29 (hPTH(29-34)).

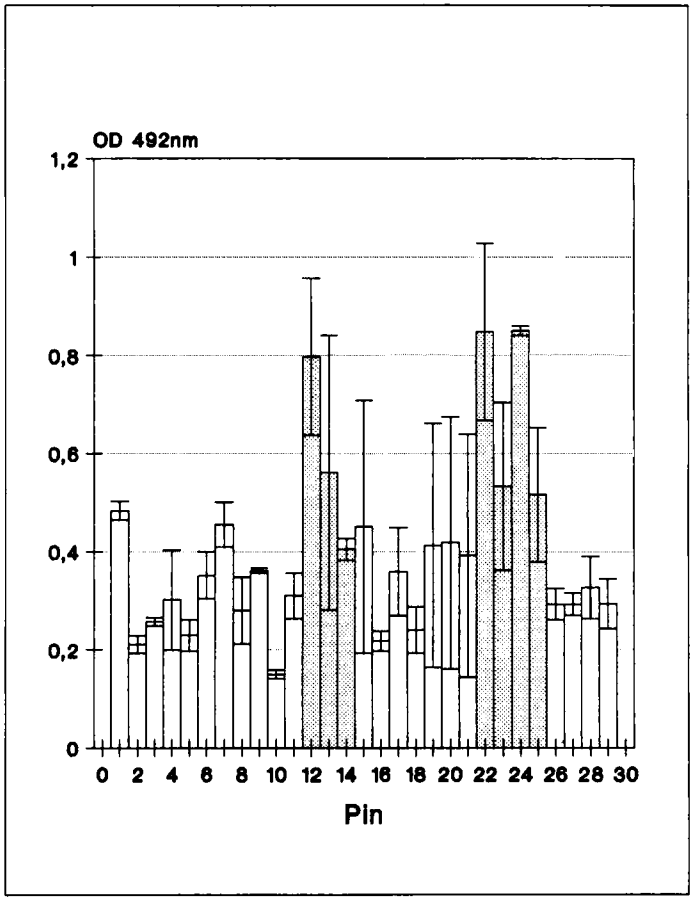

Figure 3. Binding characteristics of the monoclonal antibody A1-70 against hPTH(1-38) determined by epitope mapping. y-axis: optical density at 492 nm, maximum value, lowest value, mean value (beam), binding sequences marked. x-axis: hexapeptide on pin 1 (hPTH(1-6)) to hexapeptide on pin 29 (hPTH(29-34)).

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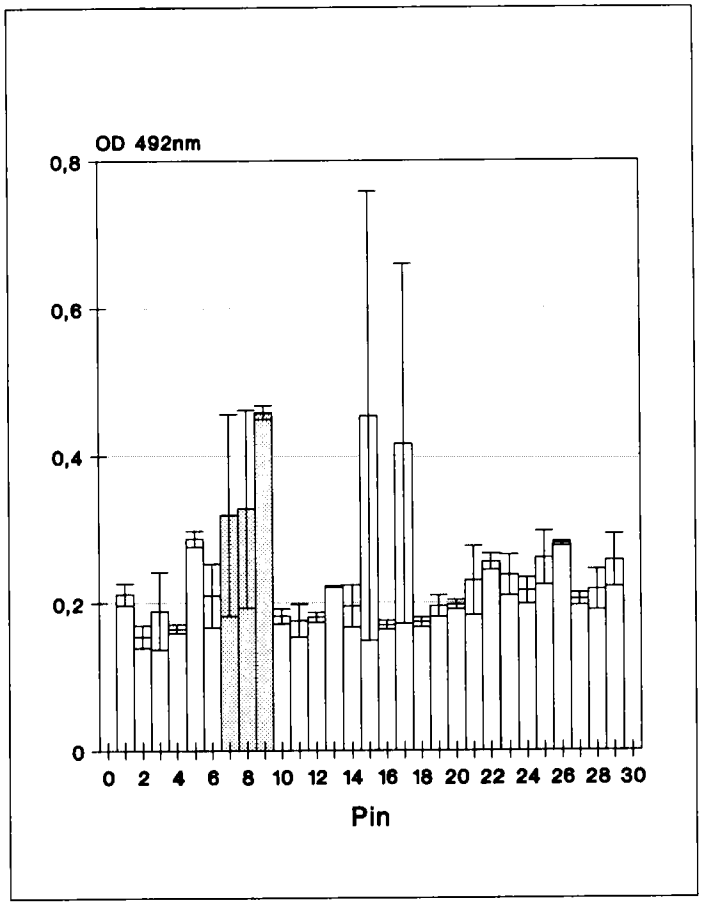

Figure 4. Binding characteristics of the monoclonal antibody A1-64 against hPTH(1-38) determined by epitope mapping. y-axis: optical density at 492 nm, maximum value, lowest value, mean value (beam), binding sequences marked. x-axis: hexapeptide on pin 1 (hPTH(1-6)) to hexapeptide on pin 29 (hPTH(29-34)).

Leu-Asn-Ser-Met-Glu), and 22-30 (pin 22-25, Glu-Trp-Leu-Arg-Lys-Lys-Leu-Gln-Asp). By contrast, the monoclonal antibody A 1-64 bound to a single epitope within the sequence 7-14 (Fig. 4; pin7-9, Leu-Met-His-Asn-Leu-Gly-Lys-His).

DISCUSSION

Epitope mapping allowed a precise and rapid characterization of binding sites of our antibodies against N-terminal PTH. The binding epitopes were localized more precisely than it was possible by the use of binding and displacement studies even with a large number of peptide fragments. In addition, this technique does not require labeling of the antigenic peptide, which may well alter binding characteristics.

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The epitope mapping kit allows the production of multiple small solid phase-bound peptides in any laboratory. The method is, however, laborious and has to be performed with great care. Even then, some "outliers" did occur (e. g. pins 15 and 17 of Fig. 4), due to the low reproducibility of the method.

A disadvantage compared with conventional approaches is that epitope mapping does not provide affinity data, and results have to be interpreted with caution as the complete peptide often has conformational changes compared with small hexapeptides. Additionally antibodies against non sequential-epitopes will not be recognized by this method.

Epitope mapping was performed to select polyclonal antisera and monoclonal antibodies directed against the N-terminal end of PTH, in order to avoid steric hindrance in two-site assays performed with

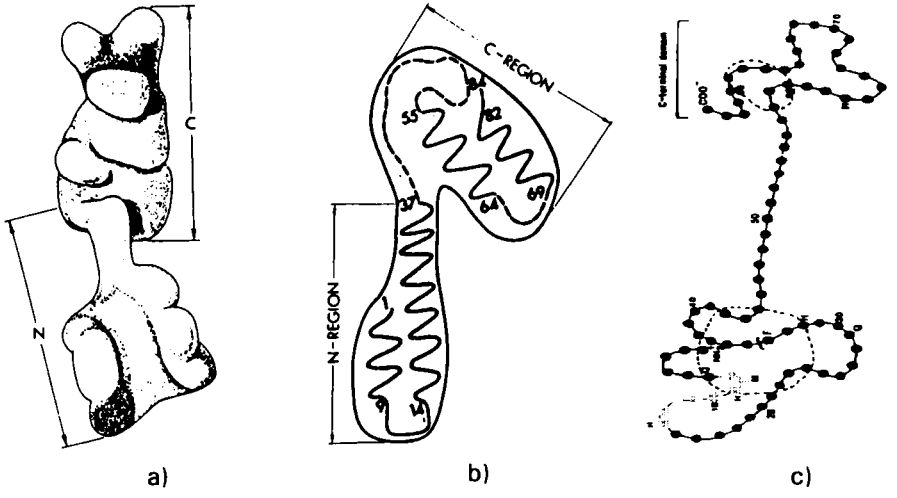

Figure 5. Three space models of bovine PTH: a): three dimensional model obtained by dark field electron microscopy (13) b): two-dimensional schematic (solid line indicates a α-helix, the dotted linea ß-pleated sheet) (see (13)) c): simplified model (14), the surface of the molecule consists of three loops which are connected by sequences building the hydrophobic core). The sequence 7-14 is marked (figures reproduced with permission).

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those antibodies (see (8)). Both the goat antiserum and the tested mouse antiserum did recognize PTH in the region 7-14 (Leu-Met-His-Asn-Leu-Gly-Lys-His). This was the epitope predominantly recognized by the goat antiserum and also recognized by one of four larger antibody subpopulations of the mouse antiserum. To obtain higher specificity with both antisera, affinity chromatography would be required. As an alternative, the monoclonal antibody A 1-64 recognizes the region 7-14 specifically.

Three dimensional models of PTH, obtained by different approaches, are in good accordance with our results: The predominant

binding region 7-14 is exposed on the surface of the molecule in all 3 models (Fig. 5).

In addition, this region seems to be easily accessible for antibodies, and our antibodies against this sequence should be useful for two-site assays of PTH and as an investigational tool.

This method provides useful guidlines to select antibodies against two apparently distinct epitopes. Nevertheless, displacement or interference of two chosen antibodies in a two-site assay my still occur. Each combination of two antibodies has to be individually testet.

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Reprints requests should be sent to Mr. F.P. Armbruster, Immundiagnostik, Wilhelmstr. 7, D 6140 Bensheim, Germany.

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