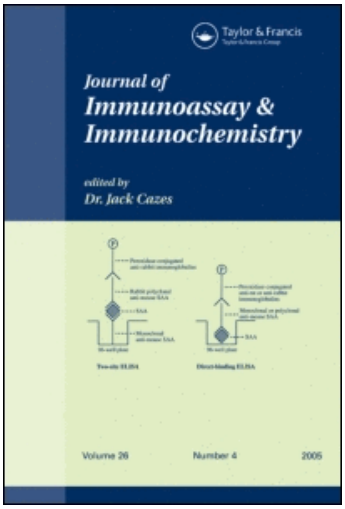

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Characterisation of the Binding Sites of Anti-Parathyroid Hormone Antisera Using Synthetic Parathyroid Hormone Peptides

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CHARACTERISATION OF THE BINDING SITES OF ANTI-PARATHYROID HORMONE ANTISERA USING SYNTHETIC PARATHYROID HORMONE PEPTIDES

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ABSTRACT

Four antisera raised against partly purified PTH preparations all showed a wide range of specificities when reacting with radioiodinated PTH peptides representing several different portions of the intact hormone sequence. In contrast, antisera raised against individual peptides were only able to cross-react with other peptides that contained all or part of their amino acid sequence in common. Cross-reacting peptides were seen to contain one or more amino acid residues having high interspecies variability in common. We have explained the antigenicity and cross-reactivity of the peptides on the basis of these common highly variable amino acid sequences. We have concluded that the selection of hormonal material in radio-immunoassays for PTH should be made on the basis of the highly variable amino acid residue content. This will allow a narowing of the assay specificities and permit detection of a desired region of the PTH hormone.
(KEY WORDS: Parathyroid hormone,Radioimmunoassay,Anti-PTH antisera, Sequence specific radioimmunoassay)

Although major portions of the amino acid sequence of Parathyroid hormone (PTH) show interspecies homology (1) sufficient sequence differences are present to render the

hormone antigenic when the species barrier is crossed (2,3). Regions of non-homology (both single and multiple substitutions) are distributed throughout the polypeptide chain in such a way that antisera selective for particular regions of the hormone can be produced (4,5,6). Indeed the present study has employed antisera reacting with h28-48,h44-68,h43-55 and h53-84 ,a range of specificities spanning almost the entire PTH sequence. Unfortunately the analytical potential of the available antisera specificities has not been fully realised. This is primarily due to the paucity of highly purified PTH peptides containing appropriate antigenic sites. Chemical synthesis of short sequences of the PTH molecule has now permitted the development of sequence-specific immunoassays in which, although multispecific antisera are generally used, each hormonal component bears an identical or closely related antigenic structure (4,5,6).

As human PTH circulates in a variety of structurally and functionally heterogeneous forms (7) tha ability to correctly assay specific regions of the hormone could be clinically important (8). Indeed the ratio of various PTH peptides within the plasma may change in direct response to certain disease conditions (9,10). Although the currently available sequence-specific assays provide a useful starting point, their future development requires a prior knowledge of the antigenic preferences of antisera raised against PTH peptides. Consequently we have set about characterising recognition sites for PTH present in a selection of anti-PTH antisera. These antisera were

raised against a variety of starting antigens, both crude extract of parathyroid tissue and synthetic short sequences of PTH. The study reveals that it is possible to predict the antigenicity of PTH peptides by determining the location and number of amino acid regions of high interspecies variability.

METHODS

Parathyroid hormone preparations

The PTH molecule contains 84 amino acids, of which there are some 13 differences between the human PTH sequence and those reported for other species (1,2,3). It is possible to group these sequence variations into small regions of structural instability (Table 1). When human PTH is used as the reference sequence it becomes evident that there are six amino acid regions where the human sequence differs from those reported for other species. We have designated these regions as ' highly variable' to indicate the unique amino acid composition of the human molecule at these positions. By using a series of PTH peptides containing one or more of these highly variable sites (Table 1) we have investigated the binding capabilities of antisera directed against both impure paratyhroid extracts and synthetic PTH peptides.

The human PTH peptides h28-48 and h44-68, and their tyrosinated derivatives, h28-48Tyr (Tyr27) and h44-68Tyr (Tyr43) were custom synthesised by solid phase methods (Bachem, Torrance,

TABLE 1

PTH PEPTIDES USED FOR THE CHARACTERISTAION OF ANTIGEN BINDING TO THE VARIOUS ANTISERA, AND THEIR CONTENT OF HIGHLY VARIABLE AMINO ACID RESIDUES

Peptide	Highly variable region contained within the peptide(Amino acid position)
h1-34	16
h28-48	40-43 46
h32-43	40-43
h43-55Tyr	46 (also contains a Tyr 42 residue)
h44-68	46 64
h53-84	64 79 83

The amino acid regions denoted above (16,40-43,46,64,79 and 83) all show a high degree of interspecies variability when hPTH is taken as the reference peptide . When a synthetic peptide contains the given highly variable region it is noted above.

Calif.). The amino (h1-34) and carboxyl (h53-84) fragments were obtained from Beckman (Paolo Alto, Calif.) and Bachem (Torrance, Calif.) respectively. The mid-regional sequences h32-43 and h43-55Tyr (Tyr42) were prepared by liquid phase synthesis by two of us (M.C. & H.Z.) (11). The purity of all synthetic PTH products was determined by reverse-phase HPLC and exceeded 95% in all cases. The partly purified human, bovine and porcine materials, and their use as immunogens are described elsewhere (12). For the tracer binding studies each synthetic peptide was

radioiodinated using Chloramine T and Iodine 125 (13).Exhaustive purification of the iodinated products was not neccessary as the labelling process included a Sephadex^{R} G-25 chromatography step. All tracers were used within one week of preparation although the tyrosine bearing peptides remain stable for many weeks after radioiodination. The h1-34 I^{125} tracer was repurified prior to use with an affinity column containing immobilized anti h1-34 antiserum (14). This was found to be neccessary to reduce the high non-specific binding of the tracer.

Anti PTH antisera

Immunisation with short fragments of hPTH was used to produce antisera specific for the antigenic domains present in selected regions of the hormone. Heterogeneous PTH preparations were used to produce antisera with broad specificities, directed primarily against the predominant antigenic material in the immunogen. All the antisera used in this study are detailed in Table 2. In each case immunisation was made using an emulsion of the antigen in Freund's complete adjuvant (Paesel, Frankfurt). The extracted PTH preparations were simply mixed with the adjuvant whereas synthetic peptides were pre-coupled to bovine serum albumin (Behring, Marburg) using carbodiimide (Sigma, München). These immunisation proceedures are described in detail elsewhere (15).

Binding assays

Antibody-antigen interactions were studied using a

TABLE 2

ANTISERA USED FOR THE CHARACTERISATION OF PTH PEPTIDE/ ANTIBODY INTERACTIONS

Antibody code	Source of Immune serum	Antigen	Major sequence specificity
As469	Sheep	Bovine and porcine parathyroid extract	Mid-regional
As478	"	" " "	"
As Giselle	"	Human parathyroid adenoma extract	"
As Claudine	"	" "	"
As Gino	"	h53-84PTH	Carboxyl terminal
As Geraldine	"	h43-55TyrPTH	Mid-regional
As Rabbit VI	Rabbit	h32-43PTH	"
As Rat 2/3	Rat	h28-48PTH	Intact

modification of the PTH-RIA technique described previously (15). The binding of tracer and the competition to this binding excerted by unlabelled PTH peptides was assessed by coincubations of immune serum and tracer at appropriate dilutions. All of the components were diluted in the 1% human serum albumin (HSA) / Barbital/ Trasylol buffer pH 7.4 (15). Antibody bound tracer was separated out using dextran-albumin-charcoal (15).

Binding of each tracer was expressed as the percentage of the total radioactivity bound by the antiserum (B/T) after correcting non-specific binding. Competition between labelled and unlabelled species was assessed under the same assay conditions. A logarithmic concentration range of each peptide

under test was added to the tracer/antibody mixture for the 24h incubation. The competition was expressed as the percentage of the tracer bound in the presence of any added peptide compared with that bound in its absence. The cross-reactivity of individual peptides was calculated by the method of Abrahams. Briefly, this compares the concentration of each peptide required to reduce tracer binding by 50% (16).

RESULTS

Antisera raised against heterologous PTH preparations

Four sheep antisera (As.469, As.478, As.Giselle and As. Claudine) were raised against crude extracts of bovine, porcine or human parathyroid tissue (Table 2). Figure1 shows that these antisera all posess the same general specificities for the synthetic PTH tracers. A high proportion of the tracer material representing amino (h1-34 I^{125}) or mid-regional (h44-68Tyr I^{125}) PTH was bound by each of these four antisera. Other tracers were bound to a lesser extent under these non-optimised conditions and reveals that sufficient antigenicity is inherent in these other regions to warrant their consideration as useful immunogens. It should be noted that the predominance of amino and mid-regional binding agrees closely with earlier studies using heterogeneous tracer material (17). The preponderance of anti mid-regional antibody probably results from the high content of 'highly variable' amino acid residues present in the h44-68

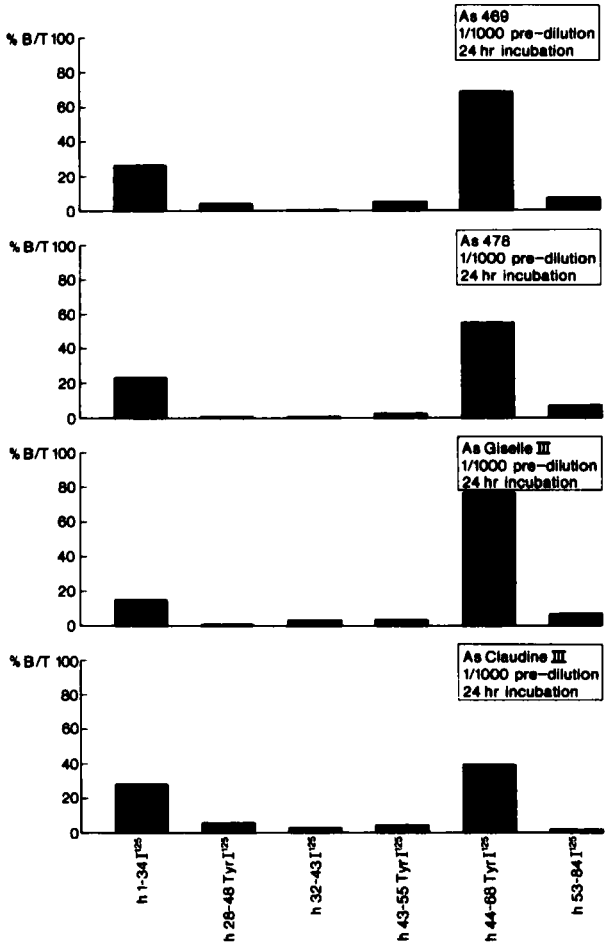


Figure 1. Tracer binding by heterologous anti- PTH antisera.

sequence (three of a possible six positions are present in the peptide).

Antisera raised against synthetic PTH peptides

1) Tracer binding

Unlike the broadly specific heterologous antisera described above, two of those raised against synthetic PTH reacted with only one tracer species. Rat 2/3 (anti h28-48) and Rabbit VI (anti h32-43) both bound the h28-48Tyr I^{125} tracer, but failed to recognise h1-34 I^{125}, h32-43 I^{125}, h43-55Tyr I^{125}, h44-68Tyr I^{125} and h53-84 I^{125} (Figure 2). The h32-43 I^{125}molecule, although containing a sequence that is identical to a major portion of that found in the h28-48Tyr I^{125} tracer, was totally inactive when incubated with either of these two antisera. This indicates that there may be some structural disruption following radioiodination of the h32-43 peptide. As there are no readily oxidizable residues in the peptide we assume that the iodo-peptide is rendered immunologically inert by the presence of the I^{125} in the molecule. The non-iodinated form of h32-43 retains immuno-activity and is able to compete with the h28-48Tyr I^{125} tracer when reacting with either Rat 2/3 or Rabbit VI (Figure 3a).

The two remaining antisera (As.Gino and As.Geraldine) each reacted with two tracer species. Thus antiserum Gino (anti h53-84) was able to bind both h53-84 I^{125} and h44-68Tyr I^{125} (Figure 2). Antiserum Geraldine (anti h43-55Tyr) reacted with both

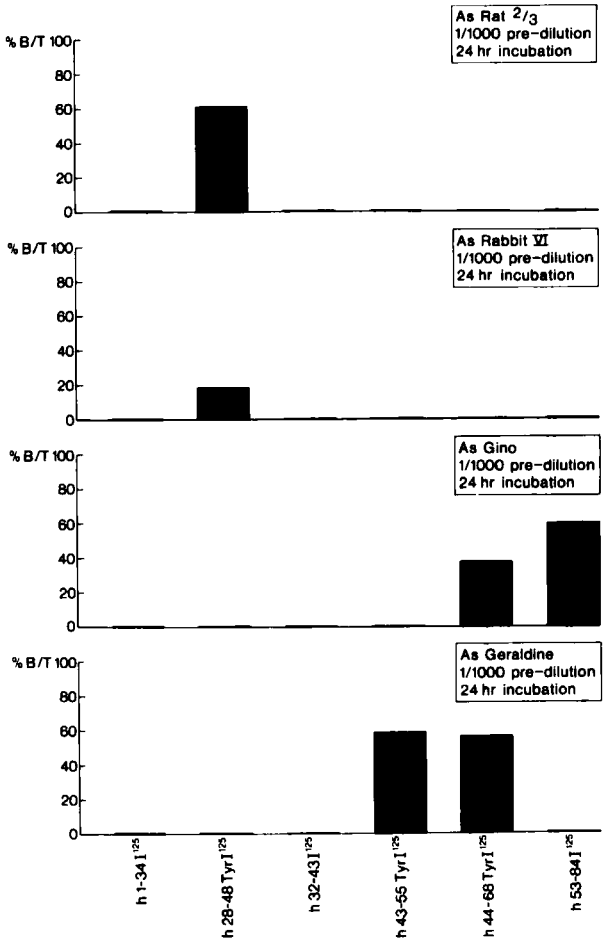


Figure 2. Tracer binding by antisera raised against synthetic hPTH peptides.

h43-55Tyr I^{125} and h44-68Tyr I^{125} tracers (Figure 2). It is evident that all of the tracer species bound by these antisera contain one or more of the six highly variable amino acid residues (see Table 1). Furthermore, when two tracers were seen to bind to one antiserum both tracers were noted to contain at least one highly variable region in common. Although such binding studies reveal the preferences of individual antisera for radiolabelled antigen, competition between uniodinated species and the tracer can define more accurately the specificities of the immune sera for different antigenic sites on the parathyroid hormone peptide chain.

2) Tracer competition

Unlabelled h28-48 reduced the binding of h28-48Tyr I^{125} to both of the antisera (Rat 2/3 and Rabbit VI) previously shown to recognise the tracer (Figure 3a and 3b). The inactivity of the radioiodinated form of the h32-43 peptide was confirmed when the unlabelled molecule was shown to compete with tracer. Indeed, h32-43 was a far stronger inhibitor of h28-48Tyr I^{125} binding to this anti h32-43 antiserum than the h28-48 peptide. Half-maximal binding occurred in the presence of 4.5 and 17.8 pMol of h32-43 and h28-48 respectively. This reveals a 25% cross-reactivity of the two peptides for the Rabbit VI and h28-48Tyr I^{125} combination. There was no appreciable influence on tracer binding when the incubation medium contained h1-34, h43-55Tyr, h44-68 or h53-84 (Figure 3a).

The binding of h28-48Tyr I^{125} to the Rat 2/3 antiserum, whilst being displacable by h28-48 was only slightly influenced

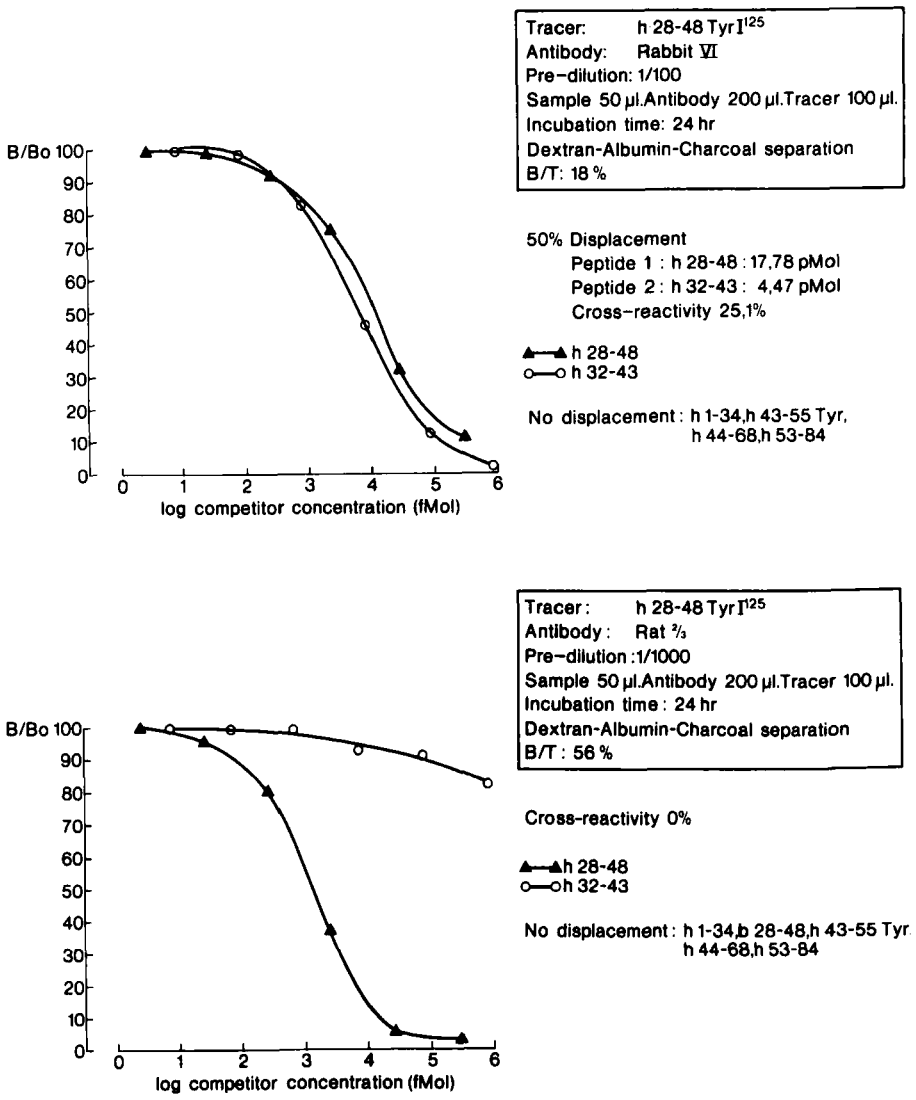


Figure 3. Competition of h28-48Tyr I^{125} tracer and unlabelled hPTH molecules for the anti hPTH32-43 antiserum Rabbit VI (Fig 3a) and for the anti hPTH28-48 antiserum Rat 2/3 (Fig 3b).

by h32-43. All other peptides tested were ineffective (Figure 3b). Significantly the bovine PTH sequence b28-48 , containing the full h28-48 sequence apart from the 5 carboxyl terminal residues, was also inactive in competition studies. This indicates that the immunological reaction is involving the carboxyl terminal residues of h28-48.

Half-maximal inhibition of h44-68Tyr I^{125} binding to the antiserum Gino (anti h53-84) was produced by either 1.3 pMol of h53-84 or 5.0 pMol of h44-68. The resultant cross-reactivity for these two peptides was approximatly 25% (Figure 4a). When the tracer was changed to h53-84 I^{125} the cross-reactivity for the two peptides was reduced to only 8%, half-maximal binding occurring at 8.9 and 112 pMol of h53-84 and h44-68 respectively (Figure 4b). In agreement with the tracer binding studies detailed in figure 2, which showed that only h53-84 I^{125} and h44-68Tyr I^{125} were recognised by this antiserum, no other peptides influenced tracer binding.

Antiserum Geraldine (anti h43-55Tyr) also showed a change in cross-reactivity when the tracer was switched. Using the h43-55Tyr I^{125} tracer half-maximal competition for binding was observed at 5.0 pMol of h43-55Tyr or 178 pMol of h44-68. This resulted in a cross-reactivity of only 3% (Figure 5a). Following a change in the tracer to h44-68Tyr I^{125} this value rose to 76%, with half-maximal binding occurring at 5.0 pMol of h44-68 and 6.3 pMol of h43-55Tyr (figure 5b). No other peptides were able to reduce the binding of either tracer to the antiserum

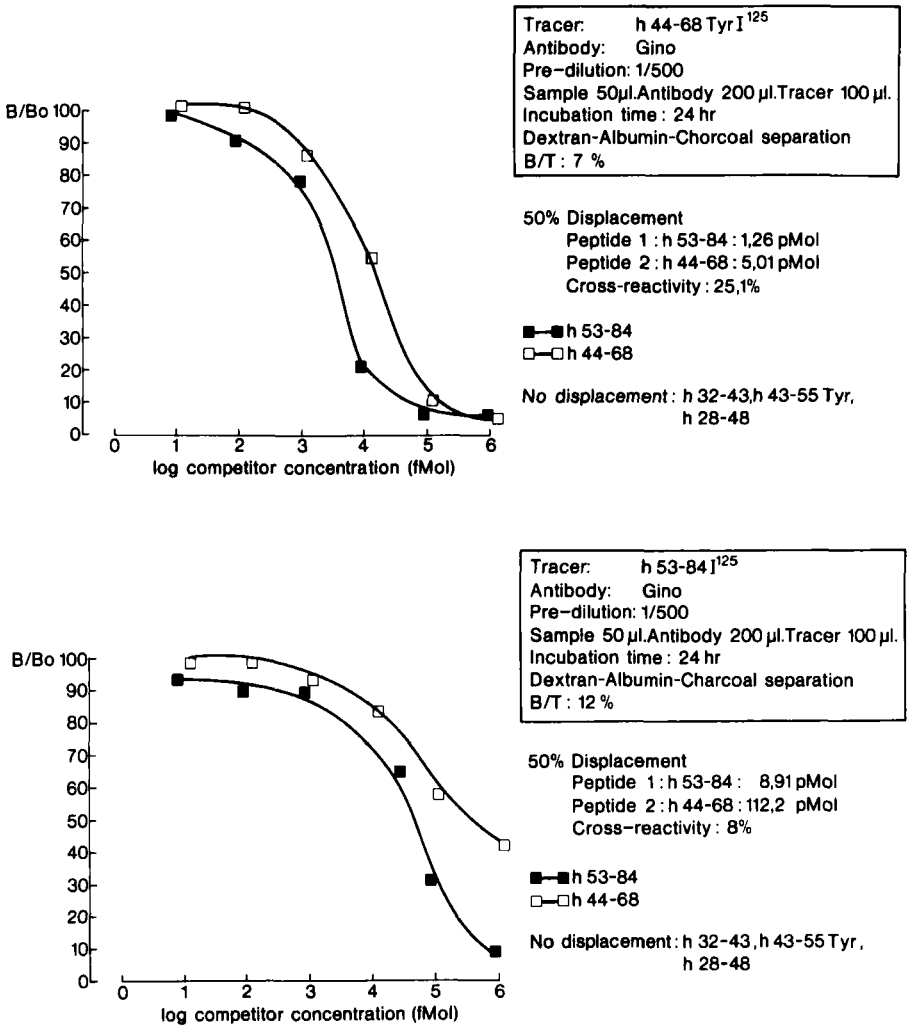
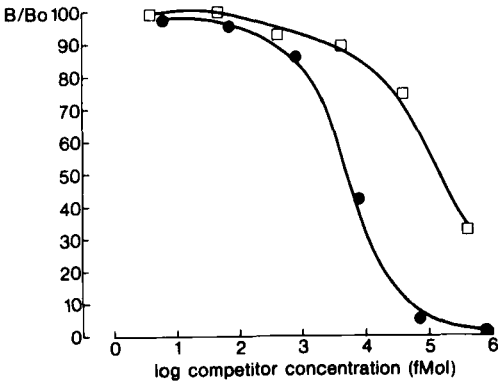


Figure 4. Competition of hPTH peptides and tracers for binding to antiserum Gino (anti h53-84): Figure 4a shows competition when the tracer was h44-68Tyr I^{125}. Figure 4b depicts the competition when h53-84 I^{125} was the tracer.

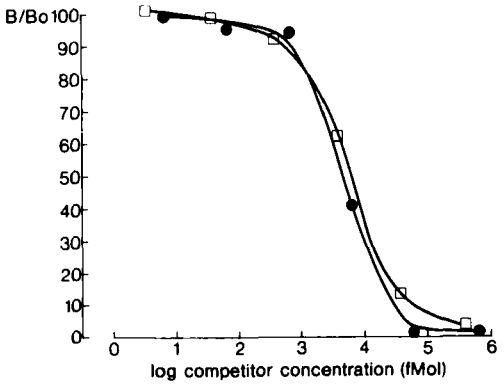


Tracer: h 43-55 Tyr I^{125}
Antibody: Geraldine
Pre-dilution: 1/1000
Sample 50 µl.Antibody 200 µl.Tracer 100 µl.
Incubation time : 24 hr
Dextran-Albumin-Chorcoal separation
B/T : 48 %

50% Displacement
Peptide 1 : h 43-55 Tyr : 5,01 pMol
Peptide 2 : h 44-68 : 177,8 pMol
Cross reactivity : 3%

●—● h 43-55 Tyr
□—□ h 44-68

No displacement : h 32-43, h 28-48, h 1-34, h 53-84



Tracer: h 44-68 Tyr I^{125}
Antibody: Geraldine
Pre-dilution: 1/1000
Sample 50 µl.Antibody 200 µl.Tracer 100 µl.
Incubation time : 24 hr
Dextran-Albumin-Charcoal separation
B/T : 23 %

50% Displacement
Peptide 1 : h 44-68 : 5,01 pMol
Peptide 2 : h 43-55 Tyr : 6,31 pMol
Cross-reactivity : 76%

□—□ h 44-68
●—● h 43-55 Tyr

No displacement : h 1-34, h 32-43, h 28-48, h 53-84

Figure 5. Competition of hPTH peptides and tracers for binding to antiserum Geraldine (anti h43-55Tyr). Figure 5a and Figure 5b show the competition when the tracer was respectively h43-55Tyr I^{125} and h44-68Tyr I^{125}.

TABLE 3

CROSS-REACTIVITY OF PTH PEPTIDES COMPETING WITH DIFFERENT TRACER SPECIES FOR THE VARIOUS ANTISERA

Antiserum	Tracer	Competing hPTH peptides	% Cross-reactivity
GINO	h53-84 I^{125}	h53-84 and h44-68	8%
	h44-68Tyr I^{125}	h53-84 and h44-68	25.1%
GERALDINE	h43-55Tyr I^{125}	h43-55Tyr and h44-68	3%
	h44-68Tyr I^{125}	h43-55Tyr and h44-68	76%
RABBIT VI	h28-48Tyr I^{125}	h28-48 and h32-43	25%
RAT 2/3	h28-48Tyr I^{125}	h28-48 and h32-43	0%

Cross-reactivity was calculated as: % Cross-reactivity= Moles of PTH peptide 1 causing 50% tracer displacement/ Moles of PTH peptide 2 causing 50% tracer displacement.

Geraldine. The cross-reactivities of the different tracer and antisera combinations are summarises in Table 3.

DISCUSSION

A series of antisera were raised against selected sequences of the human PTH molecule by immunising with synthetic hPTH peptides. The specificities of these antsiera were compared with those exhibited by antisera raised against crude preparations of human, bovine and porcine PTH. As anticipated, immunisation with

synthetic peptides produced antisera with strictly limited specificity. In a series of tracer binding studies using unlabelled PTH peptides as competing ligands it became evident that cross-reactivity between PTH peptides only occurred when certain amino acid residues were shared. In attempting to explain the cross-reactivity we noted that it was highest when the common portion of the peptide chain contained one of six regions showing high interspecies variability. When such a highly variable region was common to immunogen, tracer and each of the competing peptides cross-reactivity was high. If only one of the components lacked the shared highly variable region the cross-reactivity was reduced. In fact when there was no shared highly variable region in the two competing peptides the cross-reactivity was negligible even though other portions of the hormone sequence were shared.

Antiserum Gino (anti h53-84) was raised against a peptide containing three highly variable loci (at amino acid residues 65, 79 and 83). When the tracer h44-68Tyr I^{125} was reacted with As Gino there was a high degree of competition between the unlabelled h44-68 and h53-84 peptides. All of these hormonal components (immunogen, tracer and both competing peptides) contain the highly varaible residue at position 65. When the tracer was changed to h53-84 I^{125} the cross-reactivity was lost. This was primarily due to the inability of the h44-68 peptide to displace the h53-84 I^{125} tracer. This lack of competition is presumably due to amino acid residues present only in th h53-84 molecule. Significantly within the sequence unique to h53-84 are two

highly varaible positions (79 and 83) which would be inaccessible to the h44-68 peptide.

Although a similar series of events was observed when the antiserum Geraldine (anti h43-55Tyr) was studied, the introduction of the tyrosine molecule at position 42 may have altered the inherent antigenicity of the h43-55 sequence. Thus, when h44-68 Tyr I^{125} was employed as tracer there was a high degree of cross-reactivity between the two peptides (h44-68 and h53-84) sharing the highly variable region at amino acid 46. When the tracer was changed to h43-55 Tyr I^{125} the cross-reactivity dramatically reduced. This was due to the inability of the h44-68 peptide to displace h43-55Tyr I^{125} tracer. We propose that the introduction of the Tyr42 residue into the reaction creates a novel highly variable region accessible only to other molecules bearing the Tyr42 molecule (ie h43-55Tyr).

The reaction of Rabbit VI with the tracer h28-48Tyr I^{125} would appear to be straightforward. Both of the peptides containing the highly variable 40-43 region (h32-43 and h28-48) exhibited high cross-reactivity, whilst sequences lacking this region were inactive.

A more complicated explination is required for the reaction of antiserum Rat 2/3 (anti h28-48) with competing peptides. Only the full h28-48 sequence was recognised by the antiserum, other peptides containing large portions of the h28-48 sequence (h1-34, h32-43, h43-55Tyr and h44-68) were inactive. Moreover the bovine b28-48 peptide, containing a large amino-terminal homology with

the human sequence, was unable to displace the tracer. We conclude that no single antigenic site on the h28-48 peptide is recognised by the antiserum, and that interaction between antigenic determinants occurrs. The close proximity of the two highly variable regions 40-43 and 46 within the h28-48 peptide makes this the likely site of interaction. In the absence of a suitable peptide to test this hypothesis it remains to be proven how significant the interaction of the antigenic sites is in this antibody reaction. However, the phenomenon is not new to the field of PTH research. We have described a similar series of events when determining the specificity of the heterologous antiserum Giselle. This antiserum, when reacting with h44-68Tyr I^{125} tracer, revealed displacement characteristics of a similar nature to those described for the antiserum Rat 2/3 (6). Significantly both of these antsiera, presumably due to some form of conformational specificity, have been shown to react only with specific conformations of the human hormone when used as a plasma PTH-RIA. Thus Rat 2/3 has been shown to recognise only the intact human (h1-84) hormone, and does not display any cross-reactivity for the cleaved fragments of PTH present in blood or parathyroid tissue (18). Antiserum Giselle, in combination with h44-68 I^{125} tracer, is now known to recognise only the long-lived carboxy-terminal fragment of hPTH, and does not cross-react with the intact hPTH hormone (19).

These findings clearly demonstrate that specific antisera may be raised against synthetic sequences of hPTH, and that the

cross-reactivity may be predicted and adjusted by the appropriate choice of tracer material. We have proposed that a series of amino acid residues showing high interspecific variability occurr in all PTH peptides having immunological activity. These highly variable regions may be significant in determining the cross-reactivity of a given antiserum/ tracer combination. Consequently, by selecting hormonal material on the basis of the highly variable amino acid residue content, the specificity of clinical PTH-RIA systems may be improved and directed towards diagnostically important regions of the PTH sequence.

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