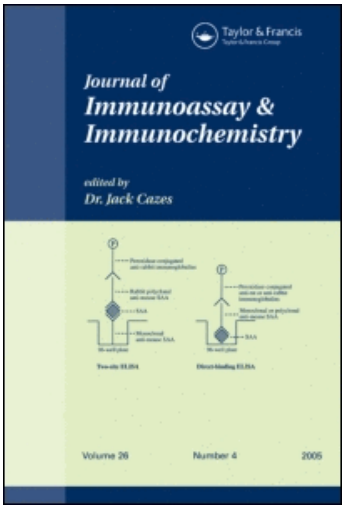

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Immune Response of the Guinea Pig to Bovine Parathyroid Hormone Antigen: Influence of the Booster Dose on Antisera Titer and Sensitivity

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IMMUNE RESPONSE OF THE GUINEA PIG TO BOVINE PARATHYROID HORMONE ANTIGEN: INFLUENCE OF THE BOOSTER DOSE ON ANTISERA TITER AND SENSITIVITY

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ABSTRACT

Thirty guinea pigs were immunized with 40 µg of bovine parathyroid hormone, bPTH(1-84), and divided into 6 groups with a similar primary response. Each group was boosted twice with a different dose of antigen (0.04 to 40 µg). The titer (30% binding of tracer) and affinity (% displacement with 320 pg of bPTH(1-84) were studied. The primary response was maximal at 61 days; titers were 1×10^{-4} or less and affinity was low. The response was maximal 25 days after each booster. After the first, the highest titers, $5.2 \pm 2.6 \times 10^{-4}$ (mean ± S.D.) were seen in the 40 µg group and a positive correlation was observed between the dose of antigen and the titer ($r = .7386$, $p < 0.001$) for doses greater than 0.6 µg. This remained true after the second booster although titers were lower in all groups. Affinity was greater after the first booster ($35.85 \pm 20.38\%$, $n = 30$) than after the second (23.24 ± 20.73, $n = 30$, $p < 0.0025$), but was similar in all groups. Selected antisera reacted with antigenic determinants in regions 1-34, 53-84 and 35-53 of the bPTH molecule. Cross-reactivity with human PTH(1-84) was maximal in the 53-84 region. In conclusion, antibodies against all regions of bPTH can be raised in the guinea pig. Once a primary response is elicited, best titers are achieved by boosting with high doses of antigen without any detrimental effect on antisera affinity.

INTRODUCTION

The production of antibodies of high titer and affinity against bovine (b) or human (h) parathyroid hormone (PTH) has been a relatively difficult task. Those people who have succeeded to get one useful antiserum (1-9) have had generally to immunize a large number of animals, if not species, with a low rate of success. It is difficult to extract from these studies clear guidance of how to proceed to obtain suitable antisera, since none of them ever provided a detailed analysis of the immune response of one animal species to the PTH antigen. Such a study is needed to successfully conceive practical smaller immunization program which will not rely on chance or animal number to get useful antisera.

Since there are many variables in the immune response, and since one cannot study them all, we decided originally to use a reliable method of immunization (10) for our program. The study was also designed to test the hypothesis that limiting doses of immunogen used as boosters may stimulate the making of antisera of high affinity by selecting antibody-forming cells with receptors of the highest affinity for the antigenic determinants of bPTH(1-84). This approach was based on the reported fact that the dose of antigen influenced the rate of maturation of antibody affinity against haptenic determinants during the primary immune response in rabbits and guinea pigs (11).

This paper describes the results that we have obtained with such an approach.

MATERIALS AND METHODS

Materials

Animals

White short hair outbreed Hartley female guinea pigs (Canadian Breeding Farm, St-Constant, Quebec), weighing ≈350 g, were kept on a normal diet supplemented with fresh vegetables and used for the immunization program.

PTH Preparations

bPTH(1-84) purified to the carboxymethylcellulose stage (12) was used for iodination. TCA-powder bPTH was purchased from the Inolex Corporation (Glenwood, Ill.) and purified by gel chromatography on BioGel P-100 (BioRad Laboratories)(12); this preparation was calibrated against bPTH(1-84) standard 71/324 in a carboxyl terminal radioimmunoassay and used for all the immunizations. bPTH(1-84) standard 71/324 was provided by the National Institute for Biological Standards (Holly Hill, Hampstead, London, NW36RB, England) as was hPTH(1-84) standard 75/549. Synthetic bPTH(1-34) was purchased from Beckman (Palo Alto, Ca. 94304); bPTH(53-84) was a gift of Dr J.T. Potts, Jr., hPTH(1-34) and hPTH (53-84) was purchased from Peninsula Inc. (611 Taylor Way, Belmont, Ca. 54002). hPTH(1-84) was obtained by extraction of pooled human parathyroid adenoma by the method of Keutman (13). This crude extract was purified by gel chromatography on BioGel P-100 (13) and calibrated against hPTH(1-84) standard 75/549 in a carboxyl terminal radioimmunoassay.

Immunization Products

Pertussis vaccine and heat killed tubercle bacilli were purchased from Institut Armand-Frappier (Laval, Quebec). Complete Freund's adjuvant was obtained from GIBCO (Grand Island, N.Y. 14072).

Methods

Immunization Program

The method of Vaitukaitis (10) was used for all the immunizations. The bPTH(1-84) antigen was first solubilized in a small amount of 0.1 M acetic acid, diluted to the appropriate volume with 0.9% sodium chloride, and mixed with an equal volume of complete Freund's adjuvant to which an additional amount of 5 mg/ml of heat killed tubercle bacilli had been added. This mixture was emulsified by passing it rapidly between two syringes connected by an adaptor. Five hundred µl of this emulsion, containing the required amount of antigen, was injected intradermally at 10-15 sites on the shaved back of each guinea pig. 0.5 ml of Pertussis vaccine was also given to half the animals at the time of the primary immunization to evaluate its effect on the primary immune response. For the booster doses, the complete Freund's adjuvant was not supplemented with heat killed tubercle bacilli and Pertussis vaccine was not used.

Thirty guinea pigs were initially immunized with 40 µg of antigen. Before the injection of the first booster, they were matched into 6 groups of 5 animals having a comparable primary

response. The first 3 groups were boosted twice with 40, 10 or 2.5 µg of antigen on day 106 and 190 while the 3 others received 0.6, 0.15 or 0.04 µg on day 148 and 239. All the animals were bled through an ear vein (14) every second week starting 30 days after the primary immunization, and every week starting 21 days after booster doses until a significant decrease in titer was observed.

Study of the antisera produced

Fifty µl of plasma coming from each bleeding of each animal was diluted to 1 X 10^{-2} with 0.05 M barbital buffer, pH 8.6, containing 10% outdated human blood bank plasma. These samples were stored at -60°C until used. Antisera were routinely assayed at a final dilution of 1 X 10^{-4}. When needed lower or higher dilutions were made with the same barbital buffer. The dilution of an antiserum giving 30% binding of the tracer was defined as the titer and this dilution was retained to measure affinity. The method of Segre (7) was used for the assay. ^{125}I-bPTH(1-84), iodinated by a modification (7) of the method of Hunter and Greenwood (15), and purified by gel chromatography on BioGel P-100 (7), was always used as tracer. Affinity was studied in non equilibrium condition with a single point type of assay; 320 pg of bPTH(1-84) or 160 pg of bPTH(1-34) were incubated for two days with each antiserum; 10,000 cpm of tracer was then added and the incubation was continued for 24 hours. For the zero point, no cold hormone was added. Damage was estimated in tubes where no antiserum was added. All procedures were done at 4°C. Final volume of the

assay was 500 µl. Barbital buffer containing 10% outdated human blood bank plasma was used to dilute all the components of the assay. One ml of 5% charcoal (Norit A) - 0.5% Dextran T 70 (Pharmacia) in barbital buffer was used to separate free from bound hormone tracer. After separation both phases were counted on a Beckman 5000 gamma counter. B/T was calculated in the usual way; $B/T = \frac{B - .D\ (B+F)}{B+F}$ where B is the tracer in the supernatant and F the tracer precipitated by charcoal, .D is the % of count behaving as bound tracer in the absence of antiserum. Percent displacement was estimated for each antiserum as being the difference between the B/T at point 0 and at point 320 pg (33.7×10^{-15} M) of bPTH(1-84).

Antisera showing maximum displacement of the tracer with cold hormone in the single point displacement assay were further studied to characterize their antigenic recognition. This was accomplished by studying the displacement of ^{125}I-bPTH(1-84) tracer bound to each antiserum by increasing amount of intact bPTH(1-84) or of synthetic fragments representative of the amino- or carboxyl-terminal sequence of the bPTH molecule. Cross-reactivity with hPTH was studied similarly with intact hPTH(1-84) or similar synthetic fragments. The assay conditions were identical to the single point assay described previously.

Statistics

Standard methods were used to calculate mean, standard deviation, linear regression, correlation coefficient and level of significance (Student's "t" test).

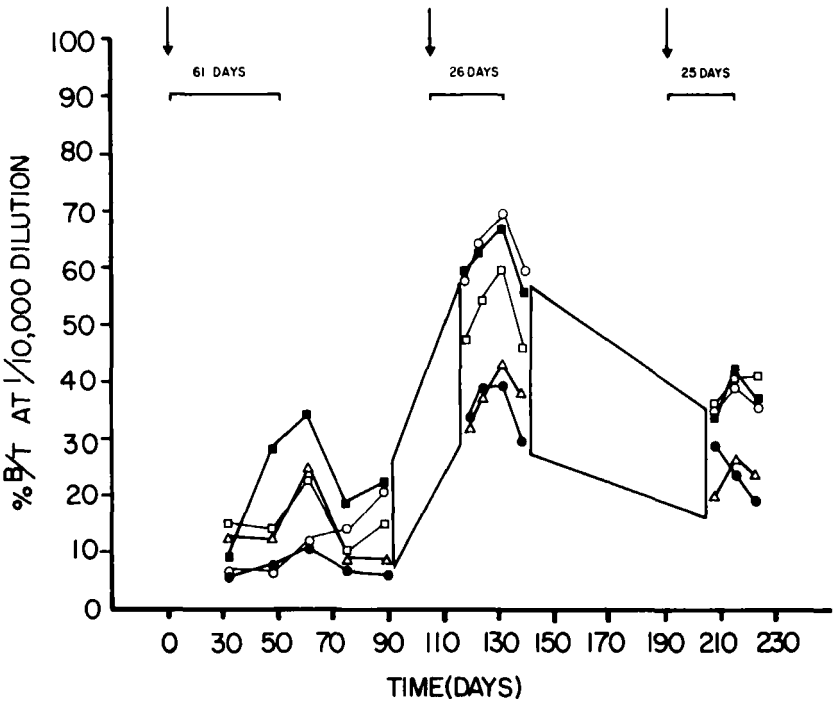

FIGURE 1 Time course study of the immune response of 5 guinea pigs to three injections of 40 µg of bPTH(1-84) antigen. The time of injection is illustrated by arrows at the top of the figure. The delineated areas represent an extrapolation of the results in periods where no sampling was done.

RESULTS

1) Time-Course Study of the Immune Response

The immune response of 5 guinea pigs who received three injections of 40 µg of bPTH(1-84) is illustrated in Figure 1. The binding capacity of each antiserum, for a fixed dilution of 1 X 10^{-4}, is maximal 61 days after the primary immunization and 25 days after each of the two booster doses. The peak of the binding capacity followed the same time-course pattern in animals receiv-

ing smaller doses of antigen as boosters (not illustrated). Further analysis and comparisons were done on the antisera collected at the peak of the primary, secondary or tertiary response.

2) Evolution of Antisera Titer

Following a primary immunization with 40 µg of bPTH(1-84), all the 30 guinea pigs developed antibodies, although titers giving 30% binding of the tracer were inferior to 1×10^{-4} in most animals. At this dilution, the mean binding capacity was $20.9 \pm 8.4\%$ (mean $\pm$ S.D.), and was similar in the 15 animals who were given Pertussis vaccine ($21.1 \pm 7.7\%$) when compared to the 15 who did not receive it ($20.7 \pm 9.3\%$).

It was possible to separate the 30 animals into 6 groups of 5 guinea pigs having a similar primary immune respone (cf. table I). After the first booster dose, those groups reimmunized with 40, 10 and 2.5 µg of antigen had an increase in their titer, while those reimmunized with 0.60, 0.15 and 0.04 µg had minimal or no increase. The highest titer of $5.2 \times 10^{-4} \pm 2.6 \times 10^{-4}$ was observed in the 40 µg group. The titers observed following the second booster dose were lower in all groups when compared with the first booster. This difference was significative for the groups who received 40 µg ($< .0125$), 2.5 µg ($< .001$), 0.15 µg ($p < 0.0025$) and 0.04 µg ($p < .0125$). As can be seen in Figure 2, a positive correlation was found to exist between the dose of antigen used as a booster and the final titer of the antisera. This was most evident in the dose range between 0.6 and 40 µg after the first booster ($r = .7836$, $p < 0.001$). Although similar

TABLE I

Immune* Response of the Guinea Pig to the bPTH(1-84) Antigen

Groups† (n=5/group)	Primary response Titer ($X\ 10^{-3}$)	Primary response B/T§ (%)	Secondary response Titer ($X\ 10^{-3}$)	Secondary response B/T§ (%)	Secondary response Affinity+ (%)	Tertiary response Titer ($X\ 10^{-3}$)	Tertiary response B/T§ (%)	Tertiary response Affinity+ (%)
40 µg	10	21.0 ±10.0	52 ±26.6	32.6 ± 2.4	33.8 ±27.7	20 ±12.3	29.8 ± 6.2	27.7 ±28.6
10 µg	10	20.0 ±11.0	36 ±19.5	31.1 ± 3.8	47.3 ± 7.1	18 ±13	28.8 ± 4.5	32.0 ±32.4
2.5 µg	10	20.1 ± 7.0	25 ± 0.0	29.4 ± 3.0	43.9 ±23.9	10 ± 0	25.8 ± 3.4	21.1 ±22.8
.6 µg	10	20.1 ± 8.9	7.0 ± 3.3	31.5 ± 5.7	26.8 ±23.0	4.4 ± 6.0	31.3 ± 6.9	19.1 ±17.8
.15 µg	10	21.3 ± 9.5	7.5 ± 2.5	31.4 ± 4.8	30.3 ±10.8	4.0 ± 1.4	28.2 ± 4.6	27.2 ± 9.4
.04 µg	10	21.6 ± 8.3	7.0 ± 3.3	27.6 ± 6.7	33.2 ±24.2	2.9 ± 2	29.4 ± 8.1	12.3 ± 4.4
(n=30)	10	20.93 ± 8.41	---	30.59 ± 4.55	35.89 ±20.38	---	28.93 ± 5.50	23.24 ±20.75

* The results illustrated correspond to the peak of the primary response at 61 days, and to the peak of the response 25 days after each booster.

† After primary immunization with 40 µg of antigen, 30 guinea pigs were subdivided into six groups of 5 animals with a comparable response and each group was boosted twice with a different dose of antigen.

§ B/T = % of cpm added bound by antisera.

\+ % of displacement obtained by the addition of 320 pg of cold bPTH(1-84).

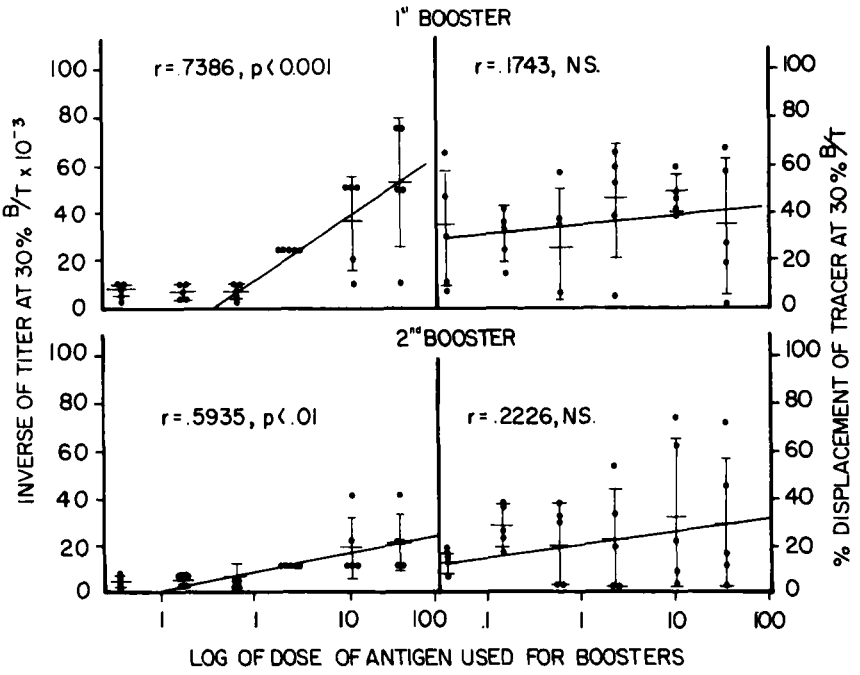

FIGURE 2 The titer and affinity of 30 guinea pigs antisera are analyzed as a function of the amount of bPTH(1-84) antigen given for booster doses. A positive correlation was found to exist between the titer of the antisera and the dose of antigen used for booster. This correlation was more evident after the first booster dose than after the second one. The affinity of the antisera remained identical at all doses of antigen used for booster.

results were observed after the second booster, the correlation was less good ($r = .5953$, $p < 0.01$).

3) Evolution of Antisera Affinity

Results are summarized in Table I and in Figures 2 and 3. After the first immunization, only an occasional antiserum demonstrated displacement of the tracer with cold bPTH(1-84) so that titers giving 30% binding of tracer were not studied. Displace-

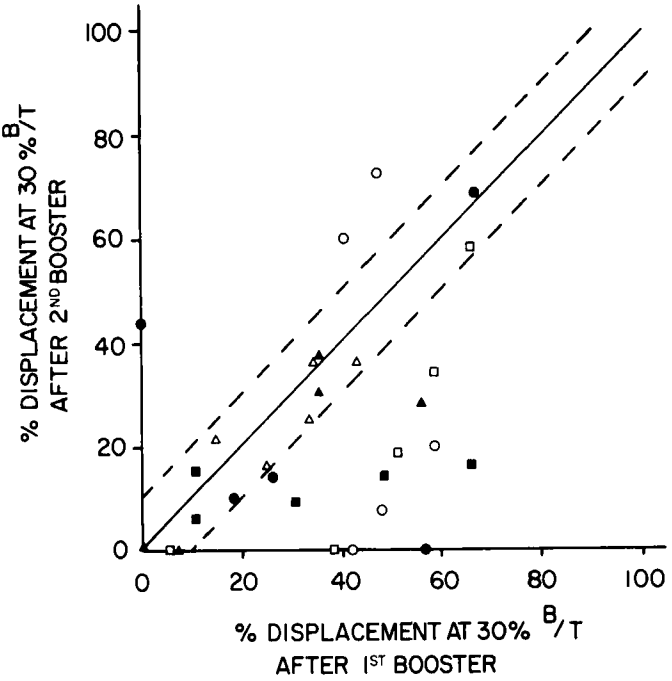

FIGURE 3 The % displacement of tracer obtained with 320 pg of bPTH(1-84) at titer giving 30% binding of the tracer is illustrated for each antiserum as a function of the two booster doses. Booster doses were 40 (●—●—●), 10 (○—○—○), 2.5 (□—□—□), 0.6 (▲—▲—▲), 0.15 (△—△—△) and 0.04 µg (■—■—■) per animal. The black line represents the response that one would have obtained if no change in affinity had been seen. Dotted lines represent a 10% deviation from this response.

ment of tracer by cold hormone was maximal 25 days after the first booster dose, even in the low doses groups where titer did not increase. The mean displacement of the tracer observed with 320 pg bPTH(1-84) was 35.89 ± 20.38% (n = 30)(range 0 to 66.9%). No relationship could be found between the dose of antigen used as a booster and the affinity of the antisera (Figure 2). This was also true after the second booster dose even if affinity

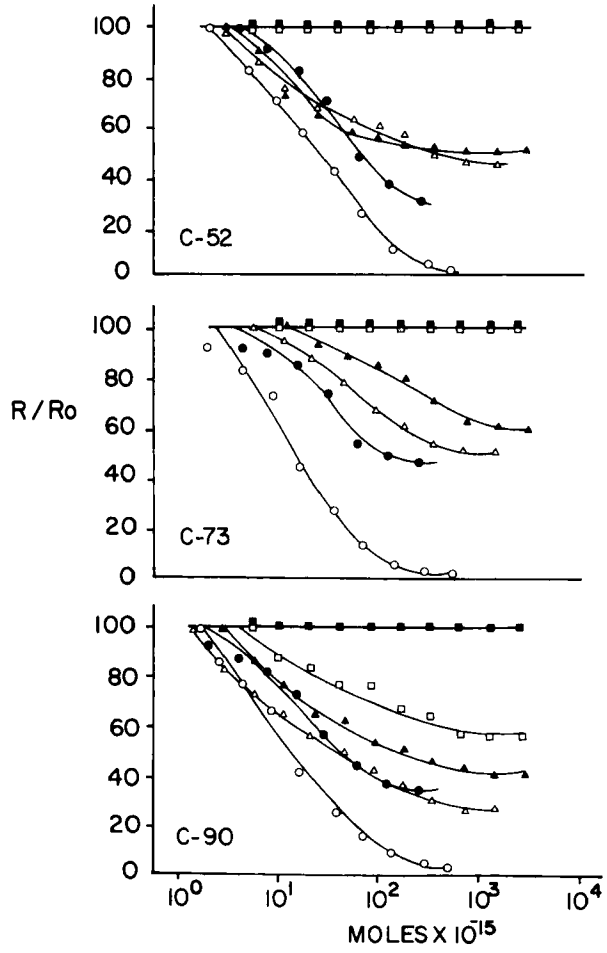

FIGURE 4 The displacement of ^{125}I-bPTH(1-84) tracer by increasing concentration of bPTH(1-84) (o—o—o), hPTH(1-84) (●—●—●), bPTH(1-34) (□—□—□), hPTH(1-34) (■—■—■), bPTH(53-84) (Δ—Δ—Δ), and hPTH(53-84) (▲—▲—▲), is illustrated for three different antisera. Antiserum C-52 was used at a final dilution of 1/25,000, C-73 at 1/37,500, and C-90 at 1/30,000. Initial B/T was near 35% in all cases.

tended to decrease in all groups. This decrease in affinity observed after the second booster dose was not significant within each group due to the small number of animals and to the large standard error but the mean displacement of tracer obtained for all groups was 23.24 ± 20.75% (range 0-72.5%) and this value was significantly lower than the one observed after the first booster ($p < 0.0025$). This is illustrated graphically in Figure 3; only 3 guinea pigs had more than a 10% increase in their % displacement of tracer after the second booster while 12 had more than a 10% decrease.

Results of displacement with 160 pg of bPTH(1-34) are not illustrated since only the occasional antiserum reacted with this region of the bPTH molecule.

4) Antigenicity of The bPTH(1-84) Molecule

Three antisera showing more than 50% displacement of the tracer with 320 pg of bPTH(1-84) were further selected to study their antigenic determinants. Results of these studies are illustrated in Figure 4.

All three antisera recognized one (or more) antigenic determinant present in the 53-84 portion of the bPTH molecule, accounting for 70% of the reactivity of antiserum C-90 and 50% of the reactivity of antisera C-52 and C-73. Since no displacement was produced by bPTH(1-34) in studies with antiserum C-52 and C-73, one has to postulate the existence of another determinant in the area 34-53 to account for the total displacement obtained with bPTH(1-84). With antiserum C-90, bPTH(1-34) dis-

placed 35% of the tracer thus the whole activity of this antiserum could be accounted for by determinants present in the 1-34 and 53-84 portions of the bPTH molecule. None of these antisera cross-reacted with hPTH(1-34), but all three cross-reacted with hPTH(53-84): but this did not account for all the carboxyl-terminal reactivity of these antisera with hPTH(1-84) and one has thus also to postulate some cross-reactivity with region 34-53 of the hPTH molecule.

DISCUSSION

Our main objective was to raise antisera of high titer and affinity against bPTH(1-84), while gaining practical informations on the immune response of the guinea pig to this particular antigen. We elected to immunize by the method of Vaitukaitis et al. (10) because it proved itself useful in raising antibodies with small doses of antigen. The only modification to their method was the omission of Pertussis vaccine in half the animals at the time of primary immunization to evaluate its effect on the immune response to bPTH(1-84). We also used progressively smaller doses of antigen for boosters in the hope of selecting antibody-forming cells with receptors of the highest affinity for the antigenic determinants of bPTH(1-84): this, we thought, would permit the raising of antisera of the highest affinity.

Following primary immunization, all guinea pigs developed significant amounts of antibodies reflecting the antigenicity of bPTH(1-84) in that species. This primary response was maximal at 61 days, a timing similar to the one observed by Vaitukaitis et

al. (10) in rabbits immunized with subunit of human chorionic gonadotrophins. This suggests that in these species the method of immunization rather than the antigen may be important for the timing of the primary immune response. Antisera titer and affinity were low after primary immunization and Pertussis vaccine had no effect on either process.

The booster doses were given 45 to 90 days after the peak of the primary or secondary immunizations when a significant fall in titer had been observed. The response to each booster followed the same kinetics and was maximal 25 days after the injection. This is slightly later than what is usually recommended for bleeding time after a booster dose (6,8,9).

After the first booster dose, the average affinity of the antisera was greatly increased in each group of animals when compared to the primary immune response. But the hypothesis that a limiting dose of immunogen given as booster might increase the affinity of the antibodies produced during the secondary immune response was not verified, contrary to what has been observed during the primary immune response (11). On the other hand, a relationship was found between the dose of immunogen used as a booster and the titer of the antisera. A booster dose of 0.60 µg or less of bPTH(1-84) caused minimal or no increase in the titer when compared with the primary response. Increasing dosage caused proportional increase in titers up to 5 fold increase with 40 µg which was the highest dose tested. This observation, though unexpected, is most interesting since it has practical implica-

tions for raising antisera of high titer in the system under study and possibly in systems with other immunogens and other animal species.

After the second booster dose, a decrease both in affinity and titer was observed when results were compared to those obtained after the first booster. Here again, there was no correlation between the dose of immunogen and the affinity of antisera, but there was a relationship between the dose and the titer. This inhibition of the response is clearly not related to the dose of immunogen since it was seen in all groups. It is not excluded that it might be related to the timing of the reimmunization, even if the booster dose was given at least 60 days after the peak of the secondary immune response.

The antisera produced shared certain characteristics. The 53-84 portion of bPTH(1-84) appears to contain the main immunogenic determinant(s) for the guinea pig and accounted for at least 50% of the reactivity of the antisera studied. This component cross-reacted in all antisera with hPTH(53-84). Another antigenic determinant, postulated to be present in the 34-53 region, was present in two of the three antisera studied and cross-reacted with hPTH. One antiserum recognized an antigenic determinant in the 1-34 region of the bPTH, which did not cross-react with hPTH(1-34). These results clearly indicate that there exist at least three antigenic determinants on the bPTH(1-84) molecule which can be recognized by guinea pig antisera.

In conclusion, bPTH(1-84) can induce an immune response in guinea pig following a primary immunization, but antisera pro-

duced are of low titer and affinity. Following a first booster with a 1000-fold range doses of immunogen, the increase of the titers of antisera is dose-dependent, while the increase in affinity is dose-independent. While both titers and affinity were lowered after a second booster, the titers were again dose-dependent and the affinity was not. These findings should be useful to those interested to start small predictable immunization programs with bPTH.

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