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SATURATION OF ANTI CYCLIC NUCLEOTIDES ANTIBODIES BY ENDOGENOUS CYCLIC NUCLEOTIDES

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ABSTRACT

Rabbits immunized against cyclic AMP or cyclic GMP produce antibodies which are fully saturated by their respective endogenous cyclic nucleotides. This was proved a) in comparing radioimmunological measurements of cyclic nucleotides in antiserum and the binding site concentration determined by equilibrium dialysis, b) in showing the ineffectiveness of serum phosphodiesterase to hydrolyze the cyclic AMP present in the anti-cyclic AMP antiserum. Immunological and radioimmunological implications of this phenomenon are discussed.

INTRODUCTION

The concentration of 3' 5'-cyclic nucleotides, cyclic AMP and cyclic GMP, in the blood represents the balance of the secretion by organs (liver, muscles, etc...) on the one hand, and of phosphodiesterase hydrolysis and kidney filtration on the other hand. Under normal conditions the turn-over of cyclic AMP, for example, is very fast and its concentration rather low, around 10^{-8} M in mammals. By studying the effect of anti-cyclic nucleotide immunization on plasmatic levels of cyclic nucleotides we hoped to answer three questions:

- Are antibodies saturated by endogenous cyclic nucleotides and, if so what effect does this have on immunized rabbits ?

- How the saturation of antibodies does affect immuno-analytical applications, namely radioimmunology ?

- What can be inferred, from an immunological point of view, from a probable interaction of antigen receptors on lymphocytes with naturally circulating ligands ?

Throughout a complete anti cyclic AMP immunization the binding capacity of antiserum, the level of cyclic AMP and the level of cyclic GMP, were systematically followed. Anti-cyclic AMP antibodies appear to be fully saturated by endogenous cyclic AMP, similar results were obtained with cyclic GMP but not with cyclic CMP.

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MATERIAL AND METHODS

Cyclic AMP-albumin conjugate (6 mole/mole) was prepared and purified as previously described (1). Four rabbits were immunized with multiple dorsal injections (2). Blood was collected in cold heparinated tube and immediately centrifuged. Part of the serum was brought to a suitable dilution and used to test the antigen's binding capacity. Another part of the antiserum was acidified with concentrated perchloric acid to 1N and left 1 h in a ice bath in order to disrupt immune complexes and to precipitate proteins. After succinylation cyclic AMP, cyclic GMP and cyclic CMP were assayed according to our standard protocols (1, 3). All measurements were performed by equilibrium dialysis (4). The concentration of antibody binding sites was determined by Scatchard analysis of displacement curves of ^{125}I-iodo-succinyl cyclic nucleotide tyrosine methyl ester by the corresponding succinyl nucleotide.

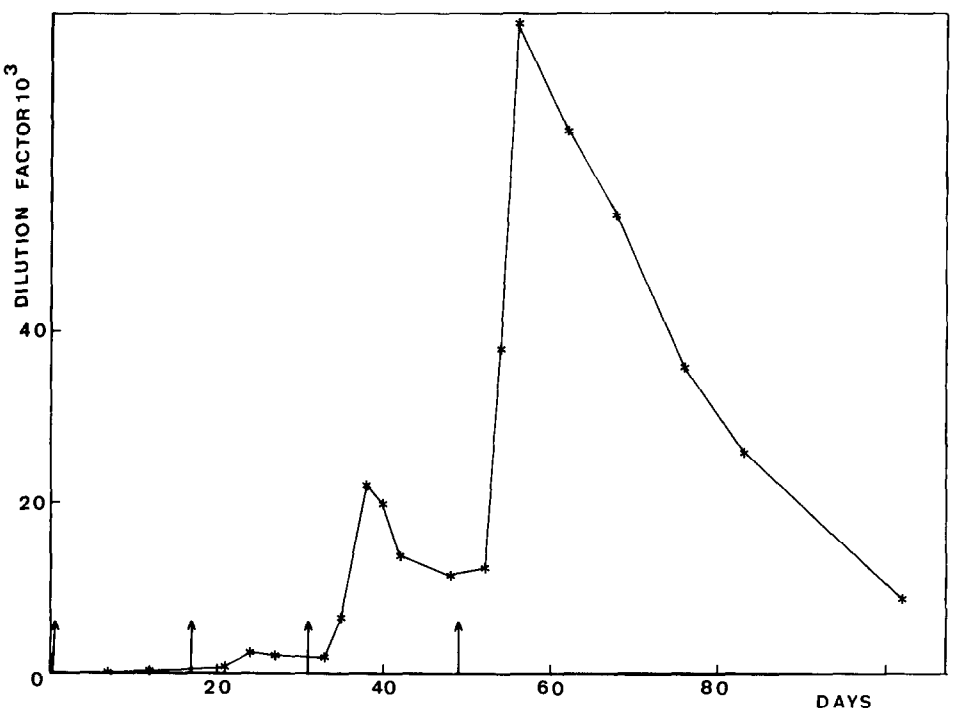

Figure 1 : Evolution of the titer of anti cyclic AMP antiserum produced by rabbit 1.
The capability of the antiserum to bind ^{125}I succinyl cyclic AMP tyrosine methyl ester was measured by equilibrium dialysis. Several dilutions were checked and the dilution which gave B/T = 0.5 was calculated. It figures on the ordinate. Arrows on the abscissa represent the booster injection.

RESULTS

Figure 1 shows the evolution of antibody titer in rabbit 1. The titer is expressed as the dilution of antiserum able to bind 50% of the iodinated derivative. This curve roughly parallels the evolution of the binding site concentration. Each injection of immunogen induced a transient increase of the antiserum titer; the maximum dilution obtained after the fourth injection reached 80,000, a rather high value for an antigen of this type. In Figure 2 are presented the concentrations of cyclic AMP and cyclic GMP measured in the same anti cyclic AMP antiserum during the immunization period. The cyclic AMP curve strongly ressembles the antibody titer curve presented in figure 1. The concentration climbed from 3×10^{-8} M at the beginning of the immunization up to 7×10^{-6} M, 7 days after the last booster injection, then slowly fell back to normal by the end of the immunization. In each peak we calculated the actual binding site concentration from a Scatchard analysis, and found it all but identical to the concentration of cyclic AMP. An

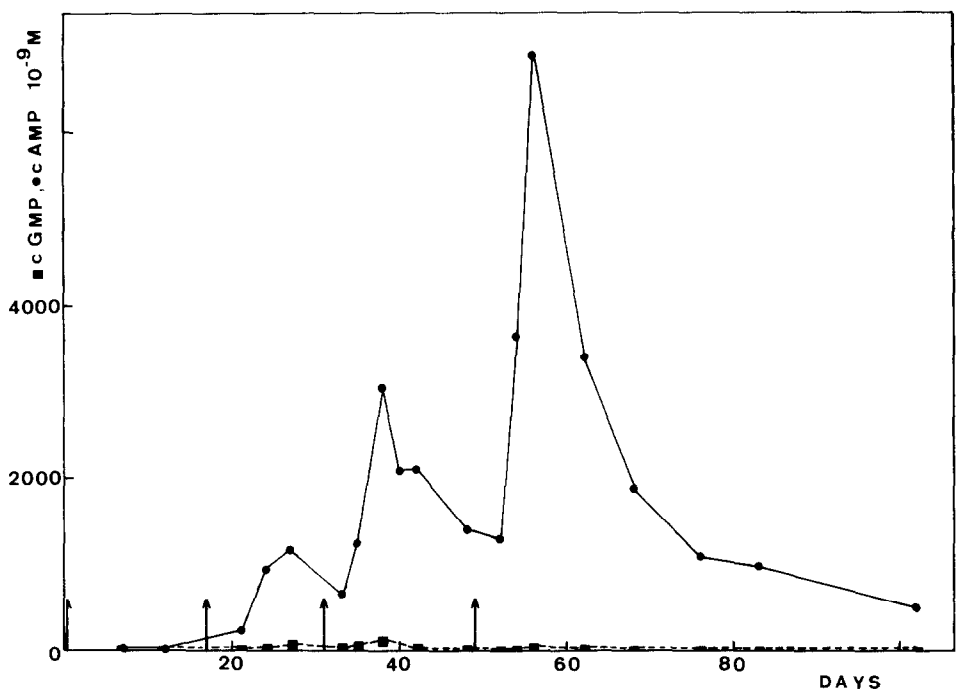

Figure 2 : Evolution of the serum level of cyclic AMP and cyclic GMP of rabbit 1 thorought the immunization period.
The blood was collected on sodium heparinate and immediately centrifuged at 4°C, 200 µl of serum were made acidic by the addition of 17 µl of $HClO_4$ 11.8 N and left for 1 hour at 4°C. Protein were discarded and cyclic nucleotides were assayed. Arrows on the abscissa indicate the booster injection.

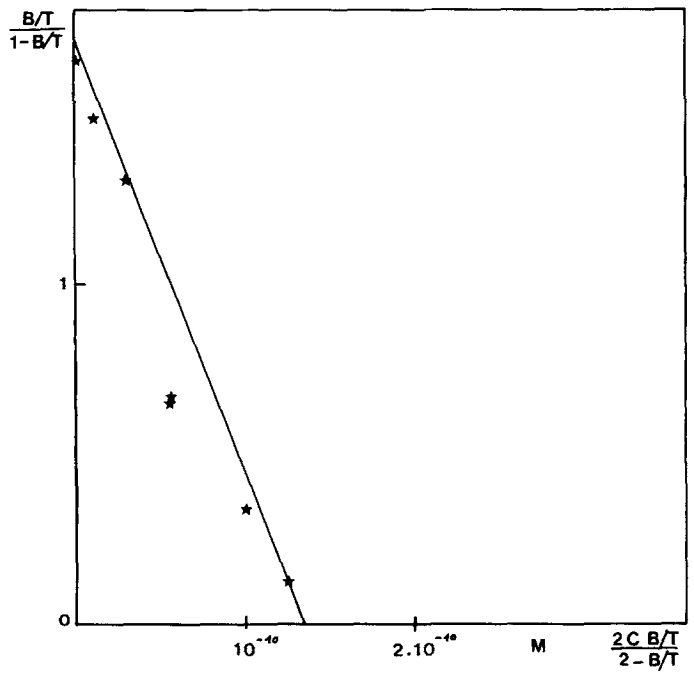

Figure 3 : Scatchard plot obtained from data of a reference curve made with the 38th day antiserum (20,400) fold diluted). Dissociation constant was K_d = 8 x 10^{-11} M. Binding sites were 2.7 x 10^{-6} M while cyclic AMP concentration was found 3 x 10^{-6} M. B = bound ; T = bound + free ; C = average concentration of ligand in dialysis. Coordinates adapted for equilibrium dialysis (see ref. 4).

example of such a calculation is given in figure 3, on the 38th day of immunization cyclic AMP concentration was 3 x 10^{-6} M and binding site concentration was 2.7 x 10^{-6} M.

The parallel behavior of concentrations of endogenous cyclic AMP and of antibody binding site strongly suggests that circulating antibodies are constantly saturated by endogenous cyclic AMP. This was confirmed by another experiment presented in Table 1. We compared the hydrolysis of cyclic AMP naturally present in antiserum with the hydrolysis of cyclic AMP added to a normal serum. The incubations were run at 20°C. At 24 h intervals aliquots of both serum were assayed for their cyclic AMP content. Practically all the cyclic AMP in the normal serum had vanished within 24 hours. In the antiserum the cyclic AMP level was quite stable and started slowly decreasing after 48 hours. Cyclic AMP is obviously protected against phosphodiesterases when bound to antibodies. Saturation of anti-cyclic AMP antibodies is due to their high affinity for cyclic AMP, characterized by a K_d value of about 10^{-8} M, and from the capability of rabbit to produce as much cyclic

TABLE I

Hydrolysis of cyclic AMP present in an anti cyclic AMP antiserum and in a control serum

	Cyclic AMP 10^{-9} M			
Incubation time at 20°C	0	24 h	48 h	72 h
38th day antiserum	3000	-	-	2400
Control serum + cyclic AMP 3000×10^{-9} M	3158	11.9	4.5	0
102th day antiserum	540	540	496	336
Control serum + cyclic AMP 1000×10^{-9} M	1191.1	3.5	2.7	0

AMP as antibody binding sites. In normal serum the rate of cyclic AMP production can be computed at between 5 and 10 x 10^{-8} M/h, assuming a first order elimination process, and using average values for cyclic AMP concentration and half-life. From our experiments we calculated that the maximal rate of production of binding sites reached at least 5 x 10^{-8} mole/liter/hour and that the maximal uptake of endogenous cyclic AMP was about 6.5 x 10^{-8} mole/liter/hour. Thus the normal metabolism apparently produces enough cyclic AMP to saturate the antibodies. However, we cannot be sure that a transient excess of free antibody did not occurred during maximum antibody production. Three of the four rabbits immunized against cyclic AMP produced antibodies which accumulated cyclic AMP. The fourth did not significantly produce antibodies and its serum level of cyclic AMP remained normal. Figure 2 also shows that the cyclic GMP level was unaffected by anti-cyclic AMP immunization, as expected from the low affinity of anti-cyclic AMP antibodies for cyclic GMP (4, 5, 6).

When immunizing against cyclic GMP the symetric phenomenon was observed: cyclic GMP concentration increased along with anti-cyclic GMP antibodies while the cyclic AMP level remained unchanged (results not shown). The situation is quite different for anti-cyclic CMP antisera: we were unable to detect cyclic CMP in serum even after a fully successful immunization. Furthermore, antisera having a strong cross-reactivity for cyclic AMP tend to accumulate cyclic AMP rather than cyclic CMP. The anti-cyclic CMP antiserum named 3C had a cross-reactivity ratio of 1/7 for cyclic AMP. Its binding sites concentration for cyclic CMP was calculated as 3.3 x 10^{-6} M and the serum level of cyclic AMP was found 7 x 10^{-8} M, a value which was three fold higher than normal.

DISCUSSION

The demonstration of the saturation of anticyclic nucleotide antibodies by endogenous cyclic nucleotides constitutes the first detailed example of what is probably a common phenomenon. Whenever an immunogen is structurally related to a circulating molecule, the antibodies raised against it can be partially or completely saturated by the endogenous ligand. This probably occurs with peptidic hormones, thyroid hormones, etc... and in some cases authentic auto-immune diseases were observed: as early as in 1956 Berson et al. (7) noted that the anti-insulin antibodies produced in patients treated with porcine insulin cross reacted with human insulin. More recently anti-vasopressin immunization was proved to induce diabetes insipidus, in human or in rabbits, by hormone deprivation (8, 9). In the case of anti-cyclic AMP immunization no physiological consequence was observed. Presumably cyclic AMP in blood is only in the process of being eliminated and does not act as a messenger.

The second question is the impact of the saturation of antibodies with immunoanalytical applications, especially radioimmunology. First, it must be recalled that anticyclic nucleotides antibodies have a much higher affinity for succinylated derivatives than for cyclic nucleotides (3, 4, 5). Thus, when they are used with ^{125}I labelled derivatives (which are succinylated) the workable dilution will bring both the binding site concentration and the endogenous concentration to around 10^{-10} M. Since the dissociation constant for non-succinylated ligands is around 10^{-8} M the endogenous ligand is no longer bound and does not interfere in competition experiments. When tritiated derivatives are used without succinylation the apparent affinity and thus the workable dilution are considerably lowered, often by two orders of magnitude. In this case, the saturation of antibodies badly influences the binding of the tracer, chiefly in non-equilibrium conditions, and a considerable underestimation of the quality of antiserum may result.

Lastly, the saturation of antibodies by endogenous ligands raises questions about how a synthetic immunogen can trigger antibody production against "self" molecules. It is generally accepted that the first event of the stimulation is the interaction of the antigenic determinant with a receptor of the IgM type. This receptor foreruns the IgG antibody which will be produce by the cell after differenciation into plasmocyte. Accordingly, we may assume that the receptors of cyclic AMP for example are also saturated by endogenous cyclic AMP. Only the receptors exhibiting a greater affinity for the immunogen, itself, i.e. recognizing the succinyl link in addition to cyclic AMP can be stimulated, resulting in antibody production. The question is now to know whether the fact that

only a sub-population of lymphocyte receptors is stimulated reduces the heterogeneity of antibodies, as compared to antibodies directed against "foreign" haptens. Work is in progress in our laboratory in order to compare the heterogeneity of the response to various haptens in correlation with their "self" or "foreign" character.

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