PRELIMINARY COMMUNICATIONS

Tolerance to High and Low Doses of Natural Crystalline Insulin

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Received: March 15, 1971, accepted: May 10, 1971

Summary. In order to examine the induction of an immunological tolerance to insulin, six different doses of once-crystallized insulin were injected i.p. into NMRI mice 3 times a week for 3 months. The doses were: 100 μ g, 10 μ g, 1 μ g, 100 ng, 10 ng and 1 ng per injection. A test immunization of 100 μ g insulin in complete Freund's adjuvant was given at the end of the immunization period to test the immunologic reactivity. It was found that one dose (1 μ g) had stimulated antibody production, and that two doses had induced tolerance: a low dose of 100 ng and a high dose of 100 μ g. In the tolerant animals at 3 and 5 weeks after the test immunization the antibody level was less than 10% of that present in the controls.

Tolérance à des doses fortes et faibles d'insuline cristalline naturelle

Résumé. Afin d'étudier l'induction d'une tolérance immunologique à l'insuline, six doses différentes d'insuline recristallisée une fois ont été injectées i. p. à des souris NMRI 3 fois par semaine pendant 3 mois. Les doses étaient de 100 μ g, 10 μ g, 1 μ g, 100 ng, 10 ng et 1 ng par injection. Une immunisation-test de 100 μ g d'insuline dans de l'adjuvant de Freund complet était administrée à la fin de la période d'immunisation, afin de tester la réactivité immunologique. Il a été trouvé qu'une dose (1 μ g) avait stimulé la

production d'anticorps et que deux doses avaient provoqué la tolérance: une faible dose de 100 ng et une forte dose de 100 μ g. Chez les animaux tolérants au bout de 3 et 5 semaines après l'immunisation-test, le taux d'anticorps représentait moins de 10% de celui des animaux témoins.

Toleranz gegen hohe und niedrige Dosen von natürlichem kristallinem Insulin

Zusammenfassung. Um die Induktion einer Immun-Toleranz gegen Insulin zu untersuchen, wurden 6 verschiedene Dosen von einmal kristallisiertem Insulin NMRI Mäusen dreimal wöchentlich über 3 Monate injiziert. Die Dosen betrugen 100 μ , 10 μ , 1 μ , 100 ng, 10 ng, 1 ng pro Injektion. Eine Test-Immunisierung von 100 ng Insulin in komplettem Freund'schen Adjuvans wurde am Ende der Immunisierungsperiode gegeben, um die immunologische Reaktivität zu prüfen. Es wurde gefunden, daß eine Dosis (1 ng) die Antikörperproduktion stimuliert hatte, und daß zwei Dosen eine Toleranz induziert hatten: Eine niedrige Dosis von 100 ng und eine hohe Dosis von 100 μ g. Im toleranten Tier betrug 3 und 5 Wochen nach der Testimmunisierung der Antikörperspiegel weniger als 10% derjenigen bei den Kontrollen key-words Immunological Tolerance to Insulin.

There exists a clinical interest to make diabetics requiring insulin tolerant to it in order to prevent complications such as the resistance to insulin caused by the formation of antibodies. By means of immunization with especially high or low doses, it is possible to get with several proteins two zones of tolerance with less than 10% of the antibody production found in normal animals, as was shown by Mitchison (1968) [3] for BSA (bovine serum albumin) in the mouse. For natural insulin, Kerp (1969) [1] obtained in the guinea-pig a partial tolerance by means of immunization with low doses of insulin, with antibody levels about 50% of those found in positive controls. Moloney (1969) [4] was able to produce a tolerance by means of modified insulin.

Obtaining a high dose tolerance to natural insulin in the same way as was done with albumin seems impossible because of the lethal effects of unphysiologically high doses of insulin. Nevertheless, we tried to employ the highest doses mice could tolerate with

the help of glucose injections. At the same time we used five other doses of insulin of ten-fold serial dilutions. To our great surprise we found both zones of tolerance after an immunization time of 3 months, 3 injections a week: a high dose tolerance and a low dose tolerance. This indicates that the doses for the induction of tolerance to insulin in our mice are much smaller than those for albumin in CBA mice, a fact that enabled the experimental demonstration of a high and a low dose tolerance to insulin in mice.

Materials and Methods

Female NMRI mice with an average weight of 30 g were randomized and separated into 7 groups of 10 mice each. They were given Altromin standard feed. A special charge of once-crystallized pig insulin was obtained from the Novowerke, Copenhagen.

The immunization was done according to the

following scheme: prior to the immunization blood samples were taken from all animals, and were used as controls for the non-specific fixation of insulin. Six groups of mice were then immunized i.p. with different doses of insulin in buffer 3 times a week for 3 months. One group of mice were given buffer only throughout. The doses of insulin were 100 μ g, 10 μ g, 1 μ g, 100 ng, 10 ng and 1 ng in phosphate buffer pH 7.8, with 1% isologous serum to avoid adsorption of insulin on the surface of the tubes. All the solutions of different insulin concentration for immunization, were made up at the beginning of the experiment, and stored at -25°C in order to keep the experimental conditions constant. Eleven days after the last immunization, blood samples were taken to examine the antibody content after the treatment. Two days later, all animals, the controls included, received a test immunization with 100 μ g of insulin in complete Freund's adjuvant at two spots under the skin, to test the immunologic reaction. Preliminary experiments showed that the antibody titre rose during the 5 weeks following the immunization with complete Freund's adjuvant. Therefore blood samples for the examination of the antibody content were taken 3 weeks and 5 weeks after the test immunization.

Our mice, although having food *ad libitum*, were not able to tolerate 100 μ g and 10 μ g of insulin. Therefore they were given 2 ml of 20% glucose s.c. one hour after the insulin injection to prevent insulin shock. Nevertheless, some animals with the highest insulin doses died after the immunization, so that we were forced to examine the survivors of the highest dose 8 weeks after the beginning of the treatment.

0.1 ml blood samples taken from the orbita of each animal were pooled with the samples of the same group and diluted to 1/20 of their volume in phosphate buffer pH 7.2 plus 0.5% EDTA. Accordingly, the serum dilution amounted to 1/40 after the separation of the erythrocytes. The antisera were stored at -25°C until the determination of the antibody content by the radioimmunoassay method of Meade [2]. 1 ml samples of the 1/40 and of the 1/80 dilution of the antisera were incubated in triplicate with 1 ml of a 1% albumin solution containing radioactive insulin at a final concentration of 3 $\mu\text{U/ml}$. The incubation was carried

and set at 0%, and the binding of the total radioactive insulin was set at 100%. In this way a percentage value for insulin binding was calculated for each serum pool. The evaluation of tolerance, however, was done with the help of controls. The antibody content of the latter after the test immunization of insulin with adjuvant was set at 100% on another percentage scale (right side of Fig. 1), and called the percentage value

Fig. 1. Low and high dose tolerance to insulin after 12 weeks of immunization. The antibody content was determined 5 weeks after the test immunization with complete Freund's adjuvant. The antibody titer of the controls was set 100% in the right scale of the graph

of the immunologic reaction. The 50% and the 10% values were obtained by dilution experiments. A reactivity of less than 50% corresponds to a partial, less than 10% to a total tolerance according to Mitchison.

Results and Discussion

After the immunization period of 3 months, the group of mice receiving the dose of 1 μ g showed a high antibody titre (Table 1). It is the same group which had the highest antibody content after the test immunization. The latter consisted of an injection of insulin plus complete Freund's adjuvant, and had been made to determine the response of the immune system to insulin. An unchanged reaction should be

Table 1

% Bound insulin after the immunization period but before the test immunization							
dose	1 ng	10 ng	100 ng	1 μ g	10 μ g	100 μ g	0 g controls
% bound insulin	5.6	6.8	5.9	90.0	10.0	—	6.1

out at 4°C in a water bath for 18 h. 300 mg of Amberlite was then added, and the amount of bound insulin was determined in the supernatant solution. For the evaluation of the antibody content, the insulin binding capacity of the sera before immunization was tested

like that of the controls and give rise to a certain amount of antibodies, which is set at 100%. A stimulated system would be expected to give more antibodies (more than 200%), and a depressed or tolerant immune system to produce no antibodies (less than

10%). All 6 doses of insulin we had been injecting for 3 months, seem to have altered the immunologic reaction to insulin. There is one stimulating dose (1 μ g) giving rise to much more antibodies than the controls (about 1600%). The other doses had a depressing effect on the immune system. Two of them, one lower than the stimulating dose (100 ng) and one higher (100 μ g), gave a depression to less than 10% of the antibody content of the controls and therefore complete tolerance. For their relation to the stimulating dose they are called low or high dose of tolerance induction.

Although the fact that the sera were pooled made it impossible to calculate statistically whether the values were significantly different, there can be no doubt of the very real difference between the effects of the stimulating dose and the two tolerogenic doses (less than 10% for the tolerogenic dose, and 1600% for the stimulating one). The high dose for tolerance induction was a little more effective than the low dose.

Two other points are remarkable. First, there is quite a rapid change from depression to stimulation when changing the immunizing dose of insulin; and secondly, it seems that as little as 1 ng of insulin when given repeatedly over a period of 3 months had a small depressing effect on the immune system.

There is a great similarity to the findings of Mitchison for the tolerance to BSA in CBA mice. He found a stimulating dose inducing more than 1000% antibodies and two depressing doses giving a tolerance with less than 10% antibodies to BSA. But there are some differences too. First, the high dose of tolerance induction was much smaller for insulin (100 μ g) than for albumin (10 mg). This fact permitted the experimental demonstration of the high dose tolerance to insulin. Higher doses of insulin were lethal for NMRI mice. The low dose of tolerance induction to

insulin was smaller (100 ng) than that for BSA (1 μ g) too. One explanation of this fact may be the ten-fold difference in molecular weight between insulin (about 6000) and albumin (about 69000). But the strain differences may also be responsible.

Our low dose tolerance found with 100 ng per mouse is about comparable with the partial low dose tolerance in the guinea-pig of Kerp. Thus he used a dose of 2 μ g/kg body weight, and we utilized in the mouse 3 μ g/kg. The two ten-fold lower doses had a tolerogenic effect in his system as well as in ours; a fact that is quite astonishing in view of the low insulin concentration.

I am very grateful to Miss H. Dorsel and Miss G. Nahler for their good technical assistance.

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