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Insulin as an antigen

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There are reviews which cover the recorded work on the antigenicity of Insulin[1-2]. This contribution is concerned principally with work done in recent years by the author and his associates.

In the early days of Insulin, in the 1920's, it was thought that Insulin was not an antigen, that it could not induce antibodies in animals or in man. This opinion continued to be generally held during the thirties and even into the early fifties, in spite of reports of *in vitro* reactions and of observations on animals and man which undoubtedly had to do with antibodies to Insulin[1-2]. Still there was the valid question: Is Insulin an antigen? Doubt regarding the antigenicity of Insulin was supported by a number of considerations: How was one to know that a given *in vitro* reaction between Insulin and a given serum was due to Insulin and corresponding antibody and not to some other antigen and antibody? In other words, was the Insulin which induced antibodies, or which was used in an *in vitro* reaction, uncontaminated by other antigen? Indeed, it was shown later that crystallized Insulin could have associated with it a non-insulin antigen which reacted *in vitro* with a corresponding non-insulin antibody[3]. Also reports of induced anaphylaxis to Insulin could be questioned for precisely the same reasons as could be *in vitro* reactions.

In respect of biological activity of Insulin, Banting, Franks and Gairns[4] had shown that serum from a man who had developed marked resistance to Insulin, when mixed with Insulin, inhibited Insulin-induced convulsions in mice. Lowell[5] also showed that blood serum from two diabetics who were resistant to Insulin could neutralize the hypoglycemic action of Insulin. Nevertheless one could question whether the antagonist in the sera responsible for neutralization of Insulin was antibody, insulinase[6], antihormone[7] or some other antagonist.

Then there was the fact that thousands of diabetics were receiving daily doses of Insulin without significant difficulty or evidence of resistance to Insulin. Furthermore, temporary resistance to Insulin could occur which was not due to neutralizing antibodies, such as during ketoacidosis. The question then—does Insulin induce antibodies?—was a reasonable one.

In our own experiments, serum from a horse which had been given a series of injections of ox Insulin in Freund's adjuvant showed a high capacity for neutralizing the hypoglycemic activity of Insulin and, when mixed with Insulin *in vitro*, showed a typical immuno reaction, namely flocculation. Furthermore, we showed that the *in vitro* reaction involved Insulin and antibodies to Insulin, since both Insulin and Insulin antibodies could be obtained in good yield from the separated and washed floccules, and that the highly purified anti-insulin extracted from the floccules showed the same *in vitro* and neutralizing properties as the untreated serum[8]. Here then was proof that Insulin was indeed an antigen.

Some characteristics of antibodies to Insulin

Antibodies which neutralize the hypoglycemic activity of Insulin can be induced in animals of various species—for example, in the guinea-pig, rabbit, sheep, horse[9-10] and man[11]—by the injection of pig or ox Insulin. The induced antibodies can neutralize not only the Insulin used for immunization (pig or ox) but also Insulins of certain other species and, in some cases, Insulin of the species in which the antibodies are induced. For example, the serum of a guinea-pig or of a horse in which anti-insulin has been induced by the injection of ox Insulin in Freund's adjuvant, when mixed with Insulin of the ox, pig, sheep, rabbit, horse, whale or mouse, can inhibit the hypoglycemic action of these Insulins. However, even though the two antisera possess the same neutralizing capacity, they are different in that the serum from the Insulin-immune guinea-pig, when injected into a living mouse, quickly induces signs of diabetes[8-9], whereas serum from the immune horse

does not[10]. This striking difference between the two antisera is most likely due to a difference in avidity or combining tendency of antibody for Insulin, since the slopes of the antibody-insulin neutralization curves for the two sera are significantly different[12].

As noted above, the injection of pig or ox Insulin into animals of certain species, including man, can induce antibodies which neutralize the hypoglycemic action of Insulin of the species injected. For example, a human diabetic, receiving Insulin, can develop, though it rarely happens, antibodies which neutralize Insulin extracted from human pancreas[5-11]. This phenomenon could indicate the termination of immunotolerance to human Insulin. It has been suggested that the induction of antibodies effective against an antigen to which an animal is immunotolerant, by the injection of a closely related antigen, represents a loss of immunotolerance[13]. If this opinion is valid, then immunotolerance to human Insulin would be terminated for a diabetic in whom ox Insulin has induced antibody-resistance to human Insulin. For such a case, treatment with human Insulin might be expected to be not only relatively ineffective but also capable of maintaining the Insulin-immunity state of the diabetic. Such a conclusion is open to question.

The validity of the hypothesis that immunotolerance to homologous Insulin can be terminated by immunizing an animal with heterologous Insulin has not yet been verified experimentally.

Sulphated Insulin

The neutralizing effectiveness of Insulin antisera toward Insulins of various species can exhibit marked differences. For example, anti-insulin induced in the guinea-pig by the injection of ox Insulin in Freund's adjuvant showed equal neutralizing capacity for Insulin of the ox, pig, mouse, sheep, horse, monkey and whale[14]; but for cod-fish Insulin[14] ten times or more antibody per hypoglycemic unit was required for neutralization, and neutralization of guinea-pig Insulin could not be detected[9-15].

The use of Insulins such as cod-fish or guinea-pig Insulins suggested itself for the treatment of diabetics showing marked antibody-type resistance to ox or pig Insulin. However, cod-fish Insulin, which could be made available, is as antigenic in guinea-pigs[16] as ox or pig Insulin and hence could be expected to serve only for short term treatment. The same could be said, most likely, for any other naturally occurring Insulin.

A study of the effect of various chemical alterations of the Insulin molecule yielded some results of interest. It was found that ox Insulin altered by the introduction of sulfate groups, required per hypoglycemic unit more antibody for neutralization than did unaltered ox Insulin[18]; in addition, the sulfated Insulin showed the surprising property of lowered antigenicity in the guinea pig[16] and in man[17]. These properties suggested that sulfated Insulin might be of use in certain cases of diabetes, and this has proved to be the case[19-20-21-22].

Induced immunotolerance to Insulin

Immunotolerance in an animal implies that no immunity response, or at most a low response, can be induced by repeated injections of a given antigen, and that the animal which is immunotolerant to a given antigen will produce antibodies to some other antigens.

Immunotolerance to certain antigens has been induced in adult mice by treatment of the mice with antigens which were non-immunogenic without adjuvant or were weakly immunogenic at certain dose levels[23-24-25].

In the study of the antigenicity of Insulin, certain observations and questions evoked an interest in the problem of induced immunotolerance, namely: anti-ox Insulin induced in the guinea-pig shows a greater binding tendency for ox Insulin than does anti-ox Insulin induced in the horse[9-10]; sulphated ox Insulin (chemically altered ox Insulin) requires more antibody for neutralization than does unaltered Insulin, which indicates a lowered combining tendency for antibody[18]; and sulfated Insulin is less antigenic than ox Insulin[17-18]. Sulfated Insulin which shows lowered antigenicity has an average of about 6 prosthetic sulfate groups per molecule, which is less than the maximum number which can be introduced. What would be the antigenicity of sulfated Insulin (or other altered Insulin) if a maximum number of sulfate groups (or other non-haptenic groups) were introduced? And what would be the characteristic of antibodies which might be induced by such altered Insulin? Since sulfated Insulin has a low binding tendency for antibodies, would it induce antibodies of low avidity? Would the particular immune system of an animal which had been taught, by pre-treatment with a chemically altered Insulin, to produce a very low level of antibodies or antibodies of extremely low avidity, be capable of producing only this type of antibody even when presented with unaltered Insulin? An animal showing such immune characteristics would be judged to be immunotolerant toward the species of Insulin in question.

These questions and speculations were put to the test by investigating first the immuno-properties of ox Insulin altered by the introduction of maleyl groups[26-27].

It was found that no neutralizing activity for either ox Insulin or for Maleyl Insulin could be detected in the serum of guinea-pigs which had received a series of injections of Maleyl Insulin, whereas the serum of control guinea-pigs injected with ox Insulin showed a marked level of neutralizing antibodies. Furthermore, guinea-pigs pre-treated with Maleyl Insulin showed only a weak immune response to repeated challenge with unaltered ox Insulin, whereas in respect of tetanus toxoid, the immune response of the pre-treated animals was the same as that of guinea-pigs which had received no pre-treatment[26-27].

Studies are under way on the effect of dosage and of chemical alteration on the immunogenic and immunotolerance-inducing properties of Maleyl and other chemically altered Insulins. It is hoped that such studies will indicate not only optimum conditions for induction of immunotolerance to Insulin, but will also throw light on the problem of induction of tolerance to other antigens.

Certain observations which bear on the question of induced immunotolerance are consistent with the assumption that the mechanisms for the induction of immunity and of immunotolerance are basically the same: for the induction of both immunity and immunotolerance an incubation period following the injection of the antigen or altered antigen is necessary; and the injection of the antigen with Freund's adjuvant markedly favors the induction of immunity and of immunotolerance.

It goes without saying that the ability to induce specific immunotolerance to any antigen in an adult animal would open new experimental approaches to many interesting problems such as acquired tolerance to viruses, bacteria and malignant cells;

hypersensitivity to foreign antigens; and the phenomenon of immuno-rejection.

Insulin serves as an excellent antigen for a study of induction of tolerance for a number of reasons namely: a great deal is known about its chemistry; adequate supplies in a state of high purity are available; Insulin shows biological activity, the neutralization of which can serve as one indicator of Insulin antibodies; diabetics with induced tolerance to Insulin would present a unique opportunity for studying the stability and duration of tolerance in the face of daily assault from injected heterologous Insulin. This problem is under active study by Dr. Alick Little, St. Michael's Hospital, Toronto.

Immunotolerance to ox Insulin in a diabetic would insure that the difficulty of increased dosage due to high antibody level would be avoided. The same end would presumably be attained by the use of human Insulin, and synthetic human Insulin has been made. However, it would be a magnificent achievement if synthetic human Insulin of even 95% purity could be made available. During synthesis, mistakes in the sequence of amino acids will be almost inevitable. So that using synthetic human Insulin for treatment of a diabetic, unless purity were 100%, one could expect the induction of antibodies to synthetic non-human peptides, and the cross reaction of such antibodies with human Insulin.

These comments are not intended in any way to pre-judge possible advantages of synthetic human Insulin for the treatment of diabetes. Undoubtedly the advantages and characteristics of synthetic human Insulin will be eventually assessed by clinical trial.

However, one can say with some confidence that in respect of induced antibodies, a diabetic rendered immunotolerant to ordinary bovine Insulin would be as well off as if treated with pure human Insulin.

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