Production of Autoantibodies to Insulin in Man and Rabbits

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SUMMARY

To determine whether antibodies to exogenous insulin bind and inactivate endogenous insulin in vivo, technics were devised to isolate and measure directly antibody-bound endogenous insulin in the presence of residual exogenous hormone in human and rabbit (leporine) serum. Insulin-antibody fractions in serum were isolated by ultracentrifugation or preferential salt precipitation, insulin was extracted by acid alcohol, and exogenous (bovine) and endogenous (human or leporine insulins) were differentially determined by immunoassay.

Insulin in the antibody fraction of normal serum is undetectable (< 10 μU. per ml.). In two resistant diabetic patients all measurable insulin proved to be bound to antibody. Exogenous insulin, which was 4 to 5 mU. per ml. seventeen hours after the last insulin treatment, remained elevated for three to sixteen days but disappeared after ten to twenty days. The subjects proved to be capable of secreting endogenous insulin that was almost completely bound to antibody in the serum. High concentrations of bound endogenous insulin (1 to 8 mU. per ml.) persisted for two to five weeks and levels were still elevated after fifty-four days. In immunized rabbits, only bound endogenous insulin was detectable three weeks after bovine insulin was discontinued. Levels remained high (1 to 2 mU. per ml.) for five weeks. Blood sugar was normal at all times, indicating that most of the bound insulin was biologically inactive.

In man and rabbits endogenous bound insulin decreased with declining antibody titers, hence did not serve as an antigenic stimulus sufficient to maintain antibody production.

Purified insulins, both human and leporine, competitively displaced bovine insulin-I-131 from the antibody sites of human and leporine serum respectively, though they were less effective than purified bovine insulin.

It is concluded that the administration of crystalline insulin can produce circulating autoantibodies capable of binding and inactivating large amounts of endogenous hormone in vivo. DIABETES *14*:396-403, July 1965.

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Moloney and Coval[1] first demonstrated that although animals treated with exogenous insulin developed antibodies, they did not become diabetic. It was presumed that the antibodies were produced only to foreign sites on the exogenous crystalline insulin and therefore did not bind the endogenous hormone. Subsequently, rabbits immunized with insulin were shown to respond normally to administered glucose and gave no evidence of pancreatic hyperactivity.[2] Though such results have been consistently observed, they do not preclude the possibility that autoantibodies* capable of binding large amounts of endogenous insulin are produced in these animals but sufficient insulin remains free to control blood sugar.

Crystalline human insulin has often been found to crossreact in vitro with antibodies from human patients treated with bovine or porcine insulin,[3-5] suggesting the formation of antibodies capable of binding endogenous human insulin. Unfortunately recent demonstrations that crystalline insulins are antigenic when injected into homologous species[6,7] indicate purified insulin may acquire unique immunologic characteristics, and therefore its binding in vitro may not reflect the binding of endogenous insulin. While immunologically detectable insulin is found associated with antibody in serum of resistant diabetic subjects after discontinuance of insulin for varying periods,[8,9] the methods used could not establish whether the insulin measured was residual exogenous or endogenous hormone.

To discover whether antibodies to exogenous insulin bind and inactivate endogenous hormone in vivo, technics were devised to isolate the insulin associated with circulating antibody in man and rabbits treated with bovine insulin and to determine immunologically what portion if any was of endogenous origin.

The methods incorporate in part a double immuno-

*Autoantibodies are defined as antibodies developed in man and animal which react in vivo with a body constituent. Such autoantibodies may arise as a result of contact of the subject with a substance related in structure to the body constituent.[24]

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assay[5] using species-specific antisera, which permit discrimination between exogenous bovine insulin and endogenous human or leporine insulin. Porcine insulin differs only by a single amino acid from human and leporine insulins, and since it reacts immunologically like the endogenous hormones with the reagent antisera, only those experimental subjects were used who had been treated or immunized with bovine insulin exclusively.

MATERIALS AND METHODS

Bovine crystalline insulin I-131 with specific activity of 3 to 5 millicuries per milligram was obtained from Abbott Laboratories. Unlabeled bovine crystalline insulin was provided by Eli Lilly and Company. Human crystalline insulin was obtained from Drs. C. N. Hales and P. J. Randle, Cambridge University, England, and Dr. R. Unger, Dallas, Texas. Crude leporine insulin was prepared from pancreas of rabbits by the acid alcohol extraction method described for human pancreatic insulin.[8]

Antiserum No. 1, prepared in guinea pigs immunized against porcine insulin, and which bound human and bovine insulin equally,[10] was used to measure total exogenous and endogenous insulin.

Antiserum No. 2, obtained from a unique, resistant diabetic subject, was previously shown to contain antibodies to bovine but not human insulin[10] and was used to discriminate between bovine and endogenous human or leporine insulin.* This antiserum, when used in the immunoassay, was less sensitive to small changes of added insulin than Antiserum 1.

Insulin-resistant diabetic human subjects

Subjects were an eighty-year-old nonobese male (Lo) and a forty-six-year-old obese female (Hi), with no family histories of diabetes, chosen for the following characteristics: (1) poorly controlled diabetes, hyperglycemia and glycosuria occurring despite treatment with 200 or more units of insulin per day; (2) high titers of circulating insulin antibody, a result of treatment with elevated levels of insulin for at least two months prior to study; (3) treatment consisting of bovine insulin only; (4) diabetes of recent onset (six years) so that residual pancreatic function was predictable; (5) no ill effects upon discontinuing exogenous insulin for more than a month.

Patient Lo was unresponsive to tolbutamide or porcine insulin.

Immunization of rabbits

One milligram bovine insulin in 0.1 mg. 0.01 N HCl was homogenized with an equal volume of complete Freund's adjuvant and injected into the toe pads of female rabbits weighing 1.5 kg. Seven and fourteen days later the animals were given a subcutaneous injection of 0.5 mg. insulin homogenized in incomplete adjuvant.

Antibody titer, the dilution of serum which bound 50 per cent of 0.1 μg. (2.5 mU.) of bovine insulin-I-131, was determined in each human or leporine serum specimen by hydrodynamic flow chromatography.[4,10]

Measurement and discrimination of circulating insulin bound to antibody

Plan 1—Measurement of total serum insulin; ultracentrifugation to determine fraction of serum insulin bound to antibody.

Insulin was extracted from serum aliquots by an acid alcohol method previously shown to dissociate and extract insulin, including that bound to antibody, with 80 per cent recovery.[8] Antibodies are precipitated during extraction, so do not interfere in the subsequent immunoassay.

Aliquots of the extracts were simultaneously immunoassayed by the method of Grodsky and Forsham,[8,11,12] in which displacement of insulin-I-131 from reagent antibody by unknown or standards is compared. Bound and free insulin-I-131 levels were determined after differential precipitation with Na_2SO_4.[11] The sole modification of the method was the use of bovine gamma globulin (3 mg. per 0.4 ml.) as carrier protein instead of the less stable alkali-treated human plasma.

Total insulin, including exogenous bovine and endogenous human, was measured with Antiserum No. 1 (figure 1). When Antiserum No. 2 was used as reagent, human insulin did not react (figure 1), and only exogenous bovine insulin was determined. The endogenous human insulin was calculated by difference. All results are means of two different dilutions, determined in duplicate and repeated on a different day. Variable nonspecific effects of the extracts on co-precipitation of the free insulin-I-131 were small and were corrected for after incubation and salt precipitation in the absence of antibody.

To establish the per cent of circulating insulin associated with antibody, human serum was diluted 1:4

*This antiserum was comparatively insensitive to porcine insulin[10] as well. Therefore exogenous porcine insulin could not be used since it was immunologically indistinguishable from endogenous human or leporine insulins.

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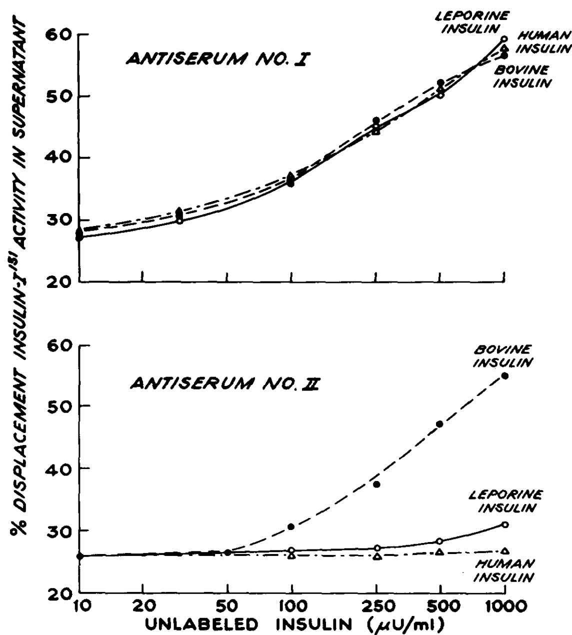

FIG. 1. Crossreaction of bovine, human and leporine insulins with antibody. Incubation mixtures contained 10 μU bovine insulin-I-131 per milliliter and either Antiserum No. 1 (diluted 1:10,000) or Antiserum No. 2 (diluted 1:200) and increasing concentrations of bovine, porcine or leporine insulins. Urea, buffer and albumin concentrations were as previously described.[8,11] Crude leporine insulin is reported in bovine insulin equivalents.

with bicarbonate buffer, pH 7.4, and centrifuged at 114,500 X g for three hours at 4° C. in a Spinco Model L ultracentrifuge. Under these conditions, all the globulins and 80 per cent of the albumins are removed from the upper 1 ml. fraction and concentrated toward the bottom of the tube.[13] Insulin bound to antibody is also quantitatively removed from the upper phase with the globulins. In the absence of antibody, regardless of the serum used, less than 25 per cent of free insulin is removed.[13] After three hours of centrifugation, the upper and lower 1 ml. fractions were isolated, insulin was extracted from each fraction with acid alcohol, and total insulin was quantitated by immunoassay with reagent Antiserum No. 1. Because of the limited supply of serum and a minimum required volume of 6 ml. in the centrifuge tubes, diluted serum was used. Minimum sensitivity of the assay of the diluted serum was 10 μU. per ml. or 40 μU. per ml. original serum.

Plan 2—Determination of bound insulin in globulin fraction isolated from serum.

Serum globulins from subject Hi or rabbits were precipitated with Na_2SO_4. The insulin in this antibody containing precipitate was extracted with acid alcohol and the human or leporine insulin determined after double immunoassay as follows:

Five milliliters serum, and 5 ml. 30 per cent urea in 1 per cent albumin were mixed and 1.7 volumes of 25 per cent Na_2SO_4 were added. After thirty minutes at 25° C. the globulin fraction was removed by centrifugation and washed once with 5 ml. 15.7 per cent Na_2SO_4 and twice with 5 ml. 5 per cent trichloroacetic acid to remove remaining salt. The precipitate was extracted twice for one hour with 20 ml. acid alcohol. The extracts were combined and the pH was adjusted with pH paper to 8.5 with NH_4OH. The crude insulin was precipitated with alcohol-ether (1.5:2.5 v/v) overnight at 4° C. and redissolved in 2.5 ml. of the glycine-albumin buffer, pH 8.6, employed in the immunoassay.[8] Table 1 shows that intact insulin-I-131, as determined by hydrodynamic flow chromatography,[14] remained in the Na_2SO_4 supernatant in normal serum, but was precipitated when antibody was present. Over-all yield of intact, originally bound insulin in the glycine-albumin buffer was 50 per cent, most of the losses occurring on the glass during the alcohol-ether precipitation in spite of a gelatin coating. Results were the same with human or leporine serum. Subsequent determinations of insulin levels were corrected for a 50 per cent recovery.

Bound exogenous (bovine) and endogenous (human or leporine) insulins were determined by the differential immunoassay. Proportional dilutions of crude leporine insulin, when assayed with reagent Antiserum No. 1, coincided with a bovine insulin standard curve (figure 1), indicating that this antiserum could be used for determining total circulating insulin in rabbits. Leporine

TABLE 1

Recovery of bound insulin-I-131 from fraction of serum containing antibody (Plan 2)

Procedural step	Recovery of insulin-I-131* (per cent)	
	Normal serum	Antiserum†
Serum + insulin-I-131	100	100
Precipitate after Na_2SO_4	0.0-14.5‡	82.2-94.5
Acid alcohol extracts of precipitate	0.0-11.7	80.0-92.1
Final alcohol-ether supernatants	0.7- 1.8	4.0- 5.6
Final alcohol-ether precipitate redissolved in glycine buffer	2.2- 3.8	47.8-53.4

*Intact insulin as determined by hydrodynamic flow chromatography.

†Normal serum + added high titer guinea pig antiserum capable of binding all the added insulin-I-131.

‡Results are range of five experiments.

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insulin was reported in beef insulin equivalents. After correcting for the slight crossreaction of leporine insulin with reagent Antiserum 2, it was possible to differentially assay the exogenous bovine insulin and the endogenous leporine insulin as described for the human studies.

RESULTS

Binding of Crystalline Insulin in vitro

Figure 2 shows antibody titers of the serum from diabetic subjects as measured by the binding of bovine insulin-I-131. In subject Lo, seventeen hours after termination of insulin treatment, titer was typical of those found in other resistant diabetics (two to fifty) in this laboratory. Levels gradually declined but antibodies were still detectable after fifty-four days. In subject Hi, levels of antibody to bovine insulin were the highest ever observed for human subjects in this laboratory; titers did not decline during the thirty-seven days after exogenous insulin was discontinued, but fell sharply thereafter.

Figure 3 shows that crystalline human insulin competitively displaced bovine insulin-I-131 from both subjects' antibodies in vitro. However, since larger amounts of human insulin were required to effect displacement its avidity for the binding sites was less than that for bovine insulin.

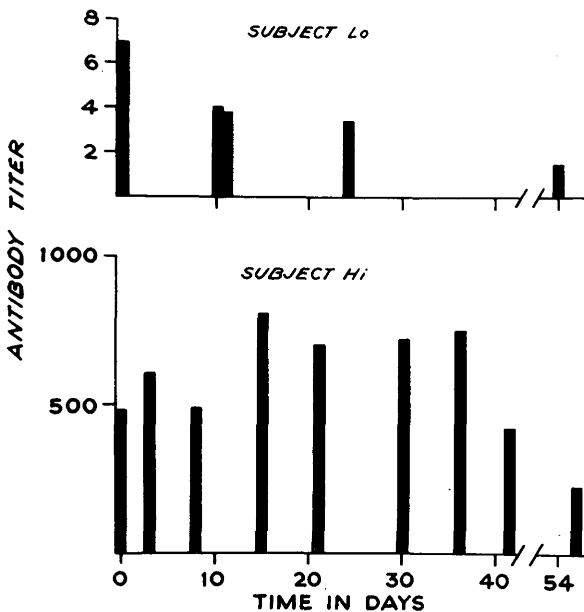

FIG. 2. Circulating antibodies to bovine insulin in diabetic subjects resistant to insulin.

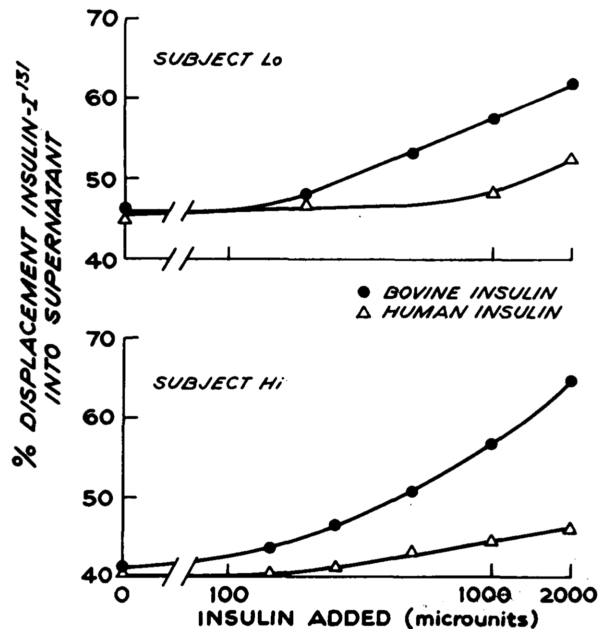

FIG. 3. Comparative displacement of bovine insulin-I-131 by crystalline bovine and human insulins from sera of resistant diabetic subjects. Serum from subject Lo was collected three days after insulin was discontinued (figure 2) and was used at a 1:100 dilution. Serum from subject Hi was collected twenty-two days after insulin was discontinued and was used at a 1:3,000 dilution.

Ultracentrifugation

Insulin-I-131 in either saline or normal serum was only slightly precipitated by ultracentrifugation, as shown by the small reduction in concentration in the upper 1 milliliter fraction (figure 4). When excess antiserum was added to the normal serum, the insulin-I-131 bound by the antibody was precipitated from the upper fraction and concentrated toward the bottom of the tube with the globulins. The result was nearly complete absence of insulin from the upper one milliliter and a concentration of approximately four times the original amount in the bottom one milliliter.

Insulin was immunologically undetectable in the extracts of supernatant fractions from all sera collected from the resistant diabetic subjects (< 10 μU. per milliliter diluted serum). In contrast, the high levels of immunologically active insulin extracted from the bottom fractions indicated that insulin had been carried toward the bottom of the tubes. Thus, in both subjects, immunologically active insulin measurable after acid alcohol extraction, whether endogenous or exogenous, was completely bound to the antibody within the limits of the experimental methods.

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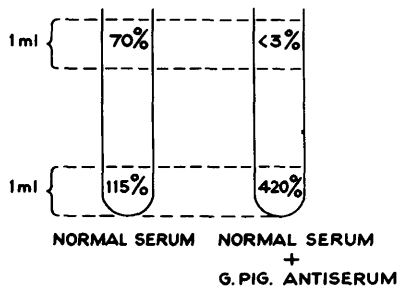

DISTRIBUTION OF TOTAL CIRCULATING INSULIN IN DIABETIC SERA*

SUBJECT	TIME AFTER INSULIN (DAY)	EXTRACTED INSULIN (μU/ml) UPPER 1 ml	LOWER 1 ml
Lo	3	< 10	1,850
	10	"	720
	11	"	700
	24	"	-
Hi	0	< 10	10,000
	3	"	9,900
	8	"	7,600
	15	"	12,500
	22	"	-
	36	"	-

* SERA WERE DILUTED 1:4 BEFORE ULTRACENTRIFUGATION

FIG. 4. Distribution of circulating insulin after ultracentrifugation of diluted (1:4) human sera.

Circulating endogenous and exogenous insulin

Mean extractable insulin levels in the serum of sixteen normal persons was 33 μU. per milliliter when assayed by the procedure used for the resistant subjects. Results of differential immunoassay of extractable insulin in the resistant subjects are shown in figure 5. In both resistant diabetics total insulin levels were extremely elevated seventeen hours after termination of insulin treatment. Levels ranged from 4.8 mU. per milliliter in subject Lo, who had the lower antibody titers, to more than 12 mU. per milliliter in Hi. In each case exogenous bovine insulin predominated. Bovine insulin attached to circulating antibody eventually declined but was measurable for three to sixteen days. After ten days in Lo and twenty days in Hi, exogenous bovine insulin was undetectable ($<$ 10 μU. per milliliter).

In both subjects, endogenous human insulin levels of approximately one to three milliunits per milliliter were found after seventeen hours, although these values were of questionable quantitative significance since they reflected a 35 per cent difference in results of two simultaneous immunoassays. After three days, however, half the bound insulin was endogenous and represented a concentration 100-350 times the normal circulating

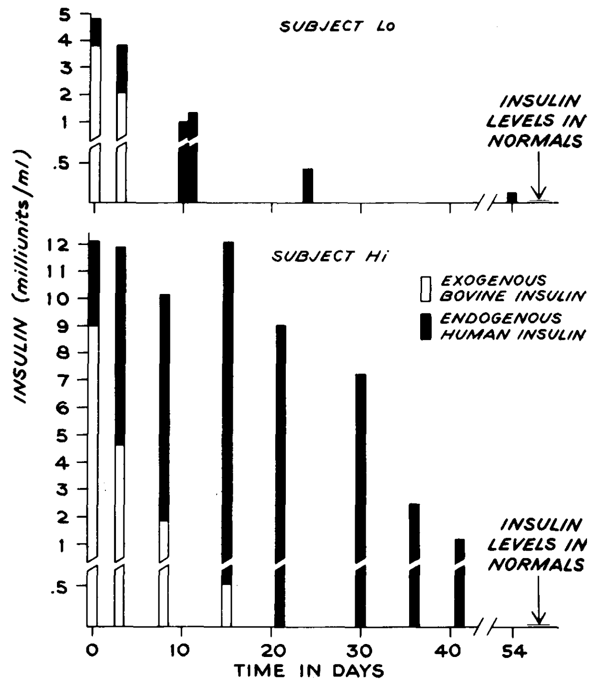

FIG. 5. Circulating total endogenous and exogenous insu in sera of resistant diabetic subjects (Plan 1).

levels. After ten days in subject Lo and twenty days Hi the bound insulin, 1 and 9 mU. per milliliter resp tively, consisted exclusively of endogenous human h mone. Endogenous bound human insulin later decrea in both subjects but was still three to fifty tin normal serum levels when the studies ended after fif four and forty-two days. In both diabetic subjects, t drop in bound endogenous insulin levels was associat with a decrease in antibody titers to bovine insulin though in Hi the decline in insulin preceded a detectal fall in antibody (figures 2 and 5).

Sufficient additional serum was available from Hi permit the isolation of the globulin fraction and t direct measurement of bound exogenous and endogen insulin by Plan 2. Results were essentially the same those obtained by assays of extracts of whole ser (figure 6).

Autoantibodies in rabbits

Antibodies to insulin, detectable before the third a final immunization dose, generally rose for two su sequent weeks (figure 7). Levels fell slowly thereaf but were still measurable eight weeks after final adm istration of exogenous insulin. Blood sugar remair normal throughout the study.

Using Plan 2, extractable insulin associated with

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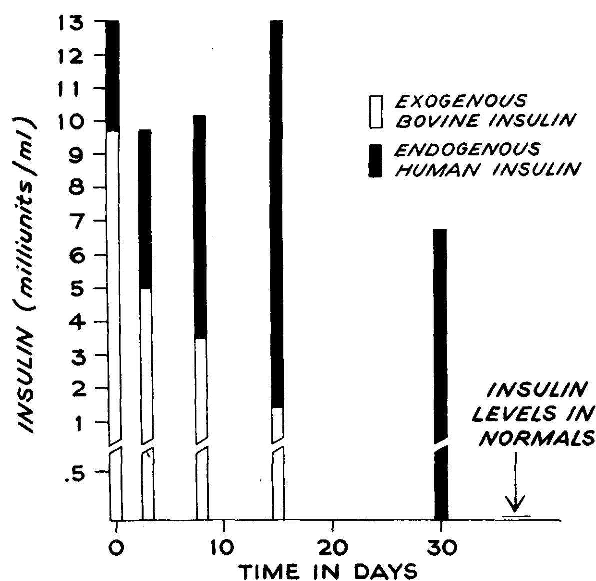

FIG. 6. Circulating endogenous and exogenous insulin associated with antibody fraction isolated from serum of subject Hi (Plan 2). Final values are multiplied by two to correct for losses during extraction (table 1).

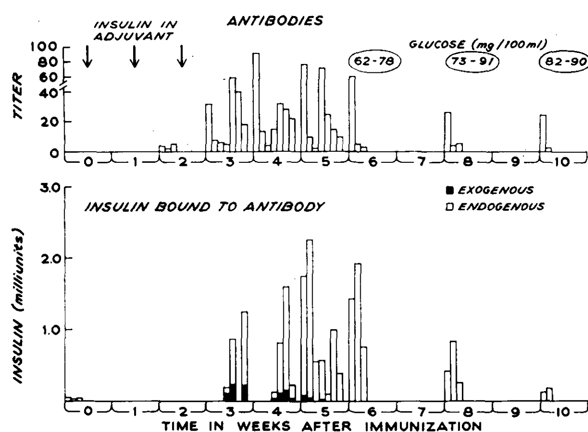

FIG. 7. Circulating endogenous and exogenous insulin associated with antibody fraction isolated from sera of seven rabbits (Plan 2). Final values were multiplied by two to correct for losses during extraction (see table 1). Nonfasting blood sugar levels are encircled. For visual clarity, exogenous insulin is reported in black.

isolated globulin fraction of normal leporine serum was immunologically undetectable (< 10.0 μU. per milliliter). Three weeks after the last immunizing dose of exogenous bovine insulin, levels of bound residual bovine insulin were too low to be detected in the presence of large amounts of endogenous leporine hormone. Bound leporine insulin was elevated (0.5-2.3 mU. per milliliter) in the globulin fraction of all but one of seven animals. The animals with the highest concentration of bound insulin did not necessarily have the highest antibody titer to bovine insulin. Endogenous bound leporine insulin decreased with the antibody titers during the subsequent weeks, but after eight weeks was still above control levels when measured as total insulin with reagent Antiserum No. 1.

In the one specimen of leporine serum with sufficient antibody to permit study, partially purified leporine insulin displaced bovine insulin-I-131 from antibody but was only 25 per cent as active as comparable concentrations of bovine insulin (figure 8).

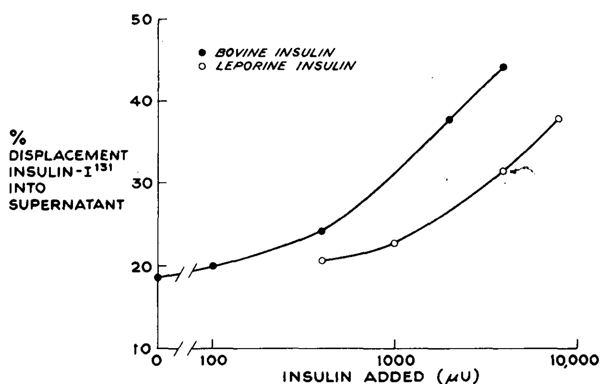

FIG. 8. Comparative displacement of bovine insulin-I-131 by crystalline bovine and partially purified leporine insulin from antiserum of an immunized rabbit. Serum was taken from the rabbit with the highest titer three weeks after final injection (figure 6) and was used at a dilution of 1:200.

DISCUSSION

The combination of ultracentrifugation or preferential salt precipitation to isolate the insulin-antibody fractions in serum, the extraction of the insulin with acid alcohol, and the differential immunoassay provided means for direct and specific measurement of antibody-bound endogenous hormone in the presence of variable amounts of bound exogenous insulin. The finding of large amounts of bound endogenous insulin attached to antibody several weeks after circulating exogenous insulin was undetectable clearly indicates that autoantibodies capable of binding endogenous hormone are produced in man and rabbits by the administration of insulin from another species.

Absolute insulin values obtained with the reagent antibodies depend on the immunologic similarity of the circulating insulins and the purified insulins used as standards. Differences between the standards and the circulating exogenous insulin are unlikely since both are commercial bovine insulin. Conceivably endogenous

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insulin may be less reactive than the human or leporine purified insulins. If so, endogenous insulin levels would actually be higher than calculated. In Plan 2, results were corrected for a 50 per cent loss during the extraction and purification procedure, based on the recovery of insulin-I-131. If endogenous insulin were fully extracted under these conditions and the correction therefore not applicable, all insulin levels would be halved but interpretation of results would still be unaffected.

The rabbits with the higher titers of antibodies to bovine insulin did not necessarily bind the largest amounts of endogenous insulin. The poor correlation of antibeef antibodies with autoantibody activity was also evident in the subject in whom binding of endogenous insulin declined even though antibody levels to bovine insulin were sustained for thirty-seven days. Both human and leporine purified insulins competitively displaced bovine insulin-I-131 from their respective antibodies, though they were less effective than purified bovine insulin. It is not yet clear whether such in vitro studies are a reliable reflection of autoantibody reaction with endogenous insulin, but if so, many of the antibody sites appear to be the same for both exogenous and endogenous insulin.

The frequency of occurrence of insulin autoantibodies in man cannot yet be determined since only two resistant subjects were available who had been treated solely with bovine insulin, had a functioning pancreas and could safely be taken off insulin. The consistent occurrence of insulin autoantibodies in rabbits, however, indicates that such autoantibodies may be a common physiological phenomenon.

The autoantibodies measured were capable of blocking the activity of bound endogenous insulin in vivo, since levels of 1-3 mU. per milliliter in the immunized rabbits did not depress the animals' blood sugar.

Despite the large quantities of insulin bound to antibody in the rabbits, adequate amounts are apparently provided to the tissues to maintain normal glucose levels and, in one report, normal glucose tolerance curves.[2] Possibly small amounts of free insulin are in equilibrium with the bound hormone. Free insulin measured after ultracentrifugation of serum diluted 1:4 could have been as high as 40 μU. per milliliter serum without being detected. Alternatively insulin may remain attached to circulating antibody and small amounts may be released at the tissue sites when required. The latter possibility is supported by Mann and Smith,[15] who found decreased insulin-like activity in sera of immunized guinea pigs.

Purified bovine insulin differs in primary structure from purified human or leporine insulin by only three amino acids. The antigen-antibody reaction, however, must involve additional sites on the molecule common to the bovine and endogenous insulin to permit occurrence of the autoantibody phenomenon.

The primary amino acid structure may contribute only in part to exogenous insulin antigenicity since purified insulin stimulates antibody production even when injected into homologous species.[5,7] Therefore, insulin's antigenicity may also arise either (1) from a modification of its secondary and tertiary structure occurring during commercial preparation or during administration or (2) by the nonphysiological route of administration (adjuvant in rabbits and slow acting insulin preparations in man) causing stimulation of an antibody response. In either instance, the antibodies could be expected to react with endogenous hormone of a similar primary structure.

In both man and rabbits, antibody titers to bovine insulin declined within six to eight weeks after cessation of insulin treatment. Bound endogenous insulin also decreased with time and with decreasing antibody. Apparently, although the immunization procedure resulted in autoantibodies reactive with endogenous insulin, the endogenous hormone itself did not become an autoantigen capable of maintaining antibody production.

A possible role of an autoimmune reaction in the etiology of diabetes has been suggested by observation of (1) histologic changes in pancreas from infants of untreated diabetic mothers, similar to changes induced by insulin antibody in animals,[16,17] (2) binding of fluorescein-tagged insulin to nodules in untreated patients with diabetic glomerulosclerosis,[18] (3) detection of a complement-consuming substance in plasma of untreated diabetics[19] and (4) diabetes in animals after passive immunization with heterologous guinea pig antisera.[20] Blumenthal et al.[21] have suggested that autoantibodies may arise from the in vivo production of abnormal insulin. Our study shows that administration of purified and possibly altered insulin can produce in man or rabbits circulating antibodies capable of binding and inactivating large amounts of endogenous hormone, a finding consistent with the very recent demonstration that administration of bovine insulin in the cow resulted in lymphocytic infiltration of the pancreatic beta cells.[22] The production of long-term diabetes in animals by an autoimmune response to exogenous insulin, heretofore unreported, has been observed in our laboratory[23] and will be the subject of a subsequent detailed report.

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