Review Articles

New Approaches to Therapy and Diagnosis of Diabetes

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Recent progress within the field of molecular biology has resulted in the development of a new technology known as 'recombinant DNA'. This technology deals with a number of biochemical techniques for handling DNA which include: (1) cutting DNA at specific sites, (2) inserting DNA into bacteria or mammalian cells so that the cell will replicate the DNA and (3) manipulation of cloned DNA so that the host cell makes the protein for which the DNA codes [1–3]. Already a number of hormones including somatostatin, human growth hormone and human insulin [4] have been produced by these new methods. However, there are certain criteria which should be considered before new sources of hormones are generally accepted for treatment of human diseases. We shall review some of the problems which may arise in the case of insulin, by comparing the chemical and immunological properties of insulin from various sources.

Recombinant DNA methods have, in addition, made it possible to study islet cell structure and function at the gene level. These studies include analysis of

gene structure and of how they are transcribed and translated. Structural analyses which seem to be significant in the differential diagnosis of diabetes will be reviewed.

Sources of Insulin (Table 1)

Insulin from Porcine and Bovine Pancreas

Modern extraction and crystallization procedures for insulin are essentially modifications of the methods used 60 years ago. Most commonly fresh or frozen pancreas is extracted using acid ethanol at pH 1–3. The extract is concentrated in vacuo, defatted, salted out, redissolved and precipitated isoelectrically at pH 5.3–5.4. The insulin is then crystallized with zinc resulting in a product which upon recrystallization exhibits constant biological activity. Insulin purified by crystallization is called conventional insulin.

Further purification by gel chromatography and ion exchange chromatography yields highly purified insulin, single component insulin or monocomponent insulin. This purified insulin is more than 99% pure; the remainder seems to represent other pancreatic hormones, such as proinsulin, glucagon, pancreatic polypeptide, vasoactive intestinal polypeptide or somatostatin. Treatment with insulin containing minute amounts of such peptides may result in the production of antibodies [9].

Insulin, a protein containing disulphide bridges and amide groups, seems to be susceptible to chemical alterations during storage. Storage of highly purified insulin for only 3 months at 4 °C may result in the formation of additional bands after polyacrylamide gel electrophoresis and components with high molecular weight demonstrable by analytical gel chromatography.

Table 1. Amino acid differences between human, porcine and bovine insulin [5–8]

	A chain			B chain
	8	9	10	30
Human	Thr	Ser	Ile	Thr
Porcine	Thr	Ser	Ile	Ala
Bovine	Ala	Ser	Val	Ala

Fig. 1. Enzymatic semisynthesis of human insulin from porcine insulin. The C-terminal alanine is removed from the porcine insulin by carboxypeptidase A at pH 8.4. Trypsin at pH 6.5 catalyzes the coupling of threonine-ester (Thr-OR) at position B-30. The ester group is removed by hydrolysis i. e. t-butyl-ester is hydrolyzed by trifluoroacetic acid (TFA)

Fig. 2. Two approaches for molecular cloning of genes to obtain bacterial production of mammalian protein products (from Riggs et al. [1])

Insulin from Human Pancreas

Human insulin has been extracted and isolated from human pancreas employing the same methods as for animal insulin [10, 11]. The crystalline human insulin is comparable to conventional porcine and bovine insulin with respect to purity as evaluated by polyacrylamide gel electrophoresis, and probably contains the same impurities as mentioned above together with degradation products formed by autolysis in the human body. The conventional crystalline human insulin has been shown to possess biological activity and antibody reactivity comparable to those of conventional porcine insulin [11].

Human Insulin by Chemical Synthesis

The first chemical synthesis of biologically active insulin was carried out in 1963 [12, 13]. A major problem in this process, apart from the correct synthesis of the A and B chains, is the combination of the two chains in the correct way. Sieber et al. [14] employed different protection groups for cysteine, enabling correct formation of the disulphide bonds. The synthetic material has been used for a few biological and clinical investigations confirming its biological activity [15–17]. However, the yield by this method is still low and gives rise to formation of several by-products.

Human Insulin from Porcine Insulin

The primary structures of human and porcine insulin differ only by one amino acid in position B30 (Table 1). Porcine insulin can be converted to insulin having the same primary structure as human insulin by exchanging the C-terminal amino acid alanine with threonine. Since insulin consists of two chains it has been difficult to limit the cleavage of the C-terminal amino acid to the B chain while sparing the C-terminal amino acid in the A chain, which is important for biological activity. This problem was solved by using enzymatic cleavage with carboxypeptidase A in specific buffer, a method which cleaves only the C-terminus of the B chain [18] (Fig. 1). Proteolytic enzymes can catalyze formation of peptide bonds [19] and thus may be used to couple threonine to des-alanine porcine insulin, resulting in a product with the same primary structure as human insulin. A variety of by-products may be formed during the cleaving and coupling procedures.

Human Insulin by Recombinant DNA Techniques

There are two strategies for producing human insulin by recombinant DNA methodologies (Fig. 2). One approach begins with messenger RNA (mRNA) and uses reverse transcriptase to make a DNA copy

(cDNA), which is then cloned into a plasmid vector. The hybrid plasmid is then used to transform bacteria. The expression of a gene will depend upon an active promoter and unless available the host bacterial cells will not be able to transcribe the foreign DNA, i.e. form a mRNA utilized for translation into the protein.

In another approach DNA corresponding to either the A or B chain was inserted into the β -galactosidase gene of the plasmid pBR 322 [4, 20]. The natural promoter of the β -galactosidase gene initiated expression of a hybrid protein (β -galactosidase-insulin A or B chain). The insulin chains were isolated by a chemical method of cleavage of the hybrid protein. The A and B chains of insulin (Fig. 3) could be isolated and the two chains linked together to form products having the same primary structure as human insulin [21]. The isolated compound was shown to possess biological and chemical identity to human insulin isolated from human pancreas [22, 23]. Possible impurities of the insulin originated from bacteria such as toxins, insulin derivatives, polymers or isomers remain to be determined.

Fig. 3. Production of human insulin from cloned synthetic pieces of DNA coding for the amino acids in the A or B chain of human insulin. The hybrid protein, consisting of part of the bacterial β -galactosidase linked by methionin to the insulin chain, is cleaved by treatment with cyanogen bromide (CNBr) (from Miller and Baxter [3])

Characterization of Insulin

Physico-Chemical Properties of Insulin

The molecular weight of insulin as estimated by gel chromatography is dependent on several factors, the most important being pH and composition of the solvent, the insulin concentration, and the metal ion content. A change in primary structure of insulin from different species might well influence the physico-chemical behaviour of the insulin molecule itself by changing the overall lipophilic character of the molecule, a theory that is supported by the fact that bovine, porcine, and human insulin can be separated by reversed phase high performance liquid chromatography [22].

It is well known that in neutral or slightly alkaline media insulin has a marked tendency to form reversible aggregates, especially in the presence of zinc ions [24–26]. When studying crystalline porcine insulin containing 0.5% zinc labelled with ^{65}Zn , employing gel chromatography on Sephadex G75 at slightly alkaline pH [27], an elution profile appeared indicating the presence of at least three separate fractions. The zinc was contained in only the high molecular weight fractions while a low molecular weight fraction (about 12,000 daltons) apparently did not contain any zinc. An increase in zinc content resulted in a displacement towards higher molecular weight, showing soluble aggregates with apparent molecular weight about 70,000–80,000 daltons and a zinc content about 1 atom/insulin monomer. In that high concentration of zinc, no zinc-free insulin was seen.

When the pH during gel chromatography was near physiological values (i.e. 7.3) and insulin with a zinc content of 0.5% was applied, most of the insulin appeared as hexamers. In order to determine the state of insulin in therapeutic preparations, analytical gel chromatography was performed on porcine insulin in a neutral buffer with a composition similar to that in the injection solution [27]. It was found that the content of zinc was a predominant factor, as a change in insulin concentration from 200 to 10 IU/ml did not change the molecular behaviour of the porcine insulin. The hexamer of the insulin seems to be a fairly stable unit which is not readily dissociated by simple dilution. When zinc is removed the hexamer breaks up and zinc-free tetramers, dimers, and monomers are formed in an equilibrium dependent on insulin concentration.

These results indicate that insulin solutions will include zinc-containing aggregates of variable sizes and zinc content, depending on pH, buffer constituent, insulin concentration, zinc concentration, and impurities. Insulin seems in addition to form poly-

Fig. 4. Immunogenicity of highly purified porcine insulin crystallized with zinc (left panel) and with the same amount of zinc added, but without crystallization (right panel). The test was performed on a solution (40 IU/ml) of the compounds (from Hansen [34])

Fig. 5. Immunogenicity of NPH-bovine insulin prepared from highly purified crystalline bovine insulin (conventional procedure) (left panel) or by direct precipitation with protamine in dissociating medium (new procedure) (right panel). The test was performed on a suspension (40 IU/ml) of the compounds (from Hansen [34])

mers of insulin molecules covalently linked together. These and other polymers are formed during the preparation and storage of porcine, bovine or human insulin. It is conceivable that the probability of these compounds occurring increases if zinc-dependent association of the type mentioned above is allowed to take place. So far no data on the physico-chemical properties of either natural or synthetic human insulin are available. Theoretically, it cannot be excluded that the processing of insulin by chemical or enzymatic synthesis, or by biosynthesis in bacteria, may result in a molecule with tertiary structure different from that in the naturally-occurring hormone.

Immunogenic Properties of Insulin

Berson and Yalow [28] reported the presence of circulating insulin-binding immunoglobulins in sera of

diabetic patients treated with insulin. It was suggested that insulin resistance and allergies were correlated with the appearance of insulin antibodies. The extensive purification of porcine and bovine insulin has reduced the prevalence of severe insulin resistance due to insulin antibodies. Nevertheless, low levels of circulating insulin antibodies are still common, although their clinical significance is still uncertain. The antigenic properties of insulin are however, sensitive tools for immunochemical characterization of insulin prepared from various sources.

It is not known how the insulin molecule is recognized by the immune system. It has been suggested [9] that impurities contained in the insulin preparations act as an adjuvant enhancing the formation of insulin antibodies [29]. Although this conclusion is still valid for conventional insulin, it cannot alone explain the remaining immunogenic behaviour of highly purified

insulin preparations. Highly purified bovine insulin is still more immunogenic than highly purified porcine insulin [30, 31]. Furthermore, homologous insulin preparations showed antibody production in cattle, sheep, goat, and man [32, 33]. A possibility exists that an antigenic stimulus has been created during or after the extraction and isolation of the insulin. This means that the antigenic determinants could be defined chemical modifications, changes in structure or formation of aggregates of the insulin molecule itself.

After subcutaneous injection, the insulin remains for an appreciable length of time in the tissue at 37 °C with a very high local concentration of insulin, especially in retarded preparations. Even though the injected insulin might have a very low content of non-dissociable polymer the condition at the injection site and during absorption may facilitate polymerization.

In order to evaluate this we have tested the immunogenicity of various highly purified porcine and bovine insulin preparations which differ in the physico-chemical state of the insulin molecule, employing the rabbit as recipient [27].

It has been possible to show that the immune response to highly purified porcine insulin in crystalline form may differ from that of the non-crystalline form (Fig. 4). This may indicate that by crystallization with zinc both reversible hexamers and higher polymers of porcine insulin are formed together with small amounts of non-reversible polymers. These phenomena could explain the difference in immune response. Such a difference was also seen in experiments performed with crystalline and amorphous forms of bovine insulin, although the levels of antibodies were much higher than those seen with the porcine preparations [34]. These findings are in accordance with the finding in patients treated with either highly purified porcine or bovine insulin [30].

We have also studied the immunogenicity of precipitated protamine insulin in rabbits and have found that when highly purified porcine insulin was employed for immunization it was not possible to detect any antibody formation in the rabbits [34]. Apparently, the binding of the porcine insulin to protamine will stabilize the porcine insulin molecule in a structure which does not permit the formation of immunogenic polymers. However, when the same test was performed with highly purified bovine protamine insulin, this was still more immunogenic than the porcine insulin preparations (Fig. 5). The tendency of bovine insulin to form aggregates before the binding to the protamine molecule is apparently too high, reflecting the more lipophilic character of the bovine insulin. In consequence of these observations we tried to isolate the bovine insulin in the monomeric state by precipitation with protamine in dissociating media just after the purification process, thus avoiding the normal iso-

lation and crystallization procedure. In this way a low immunogenic compound was actually obtained (Fig. 5).

From these studies it may be concluded that the physico-chemical properties of the insulin molecule are important for the immunogenicity of insulin preparations. If the insulin molecule is treated in a manner avoiding high concentration and crystallization with zinc, or if the insulin in the isolation phase is bound to protamine while in a monomeric state, it is possible to diminish or even eliminate the immune reaction.

Although crystalline human insulin of conventional quality has been shown to be immunogenic even in man [33], no data on the immunogenic behaviour of highly purified natural or synthetic human insulin are yet available. It is not even known whether human insulin resembles porcine and bovine insulin in immunogenic behaviour.

Recombinant-DNA Technique in the Diagnosis of Diabetes

Structure of the Insulin Gene

Using recombinant-DNA methodology, DNA fragments complementary to the mRNA of both rat and human insulin have been isolated, and the sequences determined by standard methods [35, 36]. Furthermore, these cloned insulin cDNAs were used as hybridization probes to isolate and thus allow sequencing of genomic DNA encoding rat insulins I and II as well as human insulin [37–39]. These genes contain intervening sequences (nucleotide sequences within the gene not present in the pre-proinsulin mRNA sequence) (Fig. 6). Presumably the intervening sequences are transcribed as part of mRNA precursors which are subsequently excised to form the pre-proinsulin mRNA. Pre-proinsulin mRNA then directs the synthesis of the insulin precursor, pre-proinsulin.

Localization of the Human Insulin Gene on the Chromosome

In addition to learning about the overall structure of the different insulin genes, the isolation of recombinant plasmids containing insulin DNA sequences has led to the chromosomal localization of the human insulin gene [40]. The experimental approach utilized somatic cell hybrids, formed between cultured mouse and human cells, which segregate human chromosome during cell propagation. Insulin gene sequences were examined in different cell hybrid lines using ³²P-labelled cDNA, prepared from the cloned human insulin gene. The human insulin sequence was invar-

Fig. 6. Schematic comparison of human and rat insulin genes. The coding sequences for the peptide chains of pre-proinsulin (*PRE*, *B*, *C*, *A*) are represented by the open areas. Intervening sequences (*IVS*) are indicated by the hatched areas. The numbers represent number of base-pairs (*bp*). Cap site is the first nucleotide of the primary transcript. The extent and position of nucleotide homology between intervening sequences of the two species is represented by the size of the dots – the larger the dot the greater the homology (from Bell et al. [39])

ably found when human chromosome 11 was present in the cell hybrids demonstrating that the human insulin gene is located on this chromosome. In addition, using similar techniques but including cell hybrids containing a reciprocal translocation between the short arm region of chromosome 11 and the long arm region of the X-chromosome, Owerbach et al. [41] have demonstrated that the insulin gene resides on the short arm of chromosome 11.

Restriction Fragment Length Polymorphisms of the Insulin Gene in Diabetic Patients

Another use for insulin hybridization probes will be in the detection and characterization of abnormal insulin genes [42]. Three of these genes have been identified by analysis of the abnormal proteins. Two involved the dibasic cleavage site of their proinsulin molecules and led to apparent hyperinsulinaemia [43, 44]. The third involved a substitution of a leucine for phenylalanine at either position 24 or 25 of the B chain, and was identified in a patient with hyperinsulinaemic diabetes mellitus [45]. The detection and characterization of abnormal insulin genes have been difficult, due to the scarcity of the material available for study. Furthermore, abnormal regulatory sequences contained in non-coding regions of the insulin gene cannot be detected by examination of the protein. However, the use of recombinant-DNA tech-

niques provides a basis for screening and characterizing of these abnormal sequences. Restriction fragment length polymorphisms of the insulin gene were examined in 19 Type 1 (insulin-dependent) and 16 Type 2 (non-insulin-dependent) diabetic patients [46]. DNA was isolated from lymphocyte nuclei, digested with the restriction endonuclease Bgl I, separated by size by agarose gel electrophoresis, transferred to nitrocellulose filters and hybridized with ^{32}P -labelled probe prepared from the human insulin gene. Three hybridization patterns were detected and designated U (4.5 and 5.5 kilobases), L (3.0 and 4.0 kilobases) and U, L (all four fragments). The homozygous UU genotype was present in 31% of Type 2 diabetic individuals but only in 5% of Type 1 diabetic patients examined [46]. These initial findings suggest that the UU genotype might be a genetic marker for certain forms of Type 2 diabetes.

These analyses, as well as techniques where normal or variant insulin genes are introduced into cultured cells, should increase our understanding of the genetic factors which regulate insulin biosynthesis.

Conclusion

Not only has recombinant DNA added another source of insulin for treatment, but also, and perhaps more important, it has allowed the development of new techniques for studying the structure and function of the B cell at the gene level. We can only hope that this information will bring us closer to the understanding of the disease, so the diabetic patient may benefit from other measures of treatment than the classical replacement therapy introduced by Banting and Best 60 years ago [47].

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