Proceedings of the Nutrition Society (1998), **57**, 175–181

The Summer Meeting of the Nutrition Society was held at the University of Newcastle upon Tyne on 9–11 July 1997

Plenary Lecture

Gene–environment interactions in the pathogenesis of type 2 diabetes mellitus: lessons learned from the Pima Indians

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The Pima Indians of Arizona have the highest reported prevalence of type 2 diabetes mellitus (non-insulin-dependent diabetes; NIDDM) of any population in the world (Fig. 1; King *et al.* 1993). More than half adult Pimas above age 35 years are affected (Knowler *et al.* 1990). For over 30 years, scientists from the (US) National Institutes of Health have studied this extraordinary population. The results of these investigations indicate that both acquired (environmental) and familial (genetic) factors contribute to the development of NIDDM in this population. These findings represent a unique and valuable resource for understanding the pathogenesis of NIDDM and are a lasting legacy to the invaluable contributions the Pima Indians have made over three decades.

Who are the Pima Indians?

The distant ancestors of the Pima Indians are thought to have been part of the first Paleo-Indian migration crossing the Bering land strait approximately 30000 years ago. Historical accounts and archaeological evidence indicate that the Pima Indians and their ancestors thrived in the deserts of central Arizona for more than 2000 years, building a complex system of canals to divert water from the Salt and Gila rivers for irrigation farming. In the late 19th century, settlers from the east appropriated river water for their own farming needs, wiping out many Pima farms. Gradually, a series of dams and reservoirs were built which restored water to the tribe, but not their self-sufficient agrarian lifestyle.

Diabetes mellitus was uncommon among the Pima Indians until the second half of the 20th century. In 1906, a review of the medical conditions afflicting the population listed only one case of diabetes (Hrdlicka, 1908). Elliot Joslin (1940) surveyed the tribe and, noting twenty-one cases of diabetes, concluded that the prevalence of diabetes among the Pimas was no higher than that in the general population. In 1963, an arthritis survey conducted by the National Institutes of Health unexpectedly revealed that many members of the community were diabetic. Two years later, on the basis of these findings, the National Institutes of Health initiated a formal longitudinal survey of diabetes that continues to this day. Later studies demonstrated that the Pima Indians are hyperinsulinaemic and insulin resistant relative to other ethnic groups (Aronoff *et al.* 1977; Nagulesparan *et al.* 1982) and that diabetic subjects, even those with an early age of onset, do not manifest anti-islet cell antibodies (Knowler *et al.* 1979). Thus, among the Pimas virtually all diabetes is classified as NIDDM.

What is the evidence that environmental or acquired factors contribute to type 2 diabetes mellitus in Pima Indians?

The increasing prevalence of NIDDM among the Pima Indians during this century coincided with marked and rapid changes in lifestyle. The traditional Pima diet was high in complex carbohydrates and low in saturated animal fats (Swinburn *et al.* 1991). With the decline in traditional farming, community members turned for survival to government surplus commodities, which were generally high in fat. The transition to a 'modern' diet contributed to an increase in the prevalence of obesity (Price *et al.* 1993), which led to insulin resistance and, in turn, worsening glucose tolerance (Bogardus *et al.* 1985). Among Pima Indians the incidence of diabetes is strongly related to BMI (kg/m^2), such that the risk to individuals with a BMI above 40 is 10-fold higher than that among individuals with a BMI of less than 20 (Fig. 2). Both the duration of obesity (Everhart *et al.* 1992) and the rate of weight gain (Hanson

Abbreviations: IFABP, intestinal fatty acid binding protein; NIDDM, non-insulin-dependent (type 2) diabetes mellitus; MODY, maturity-onset diabetes of the young.

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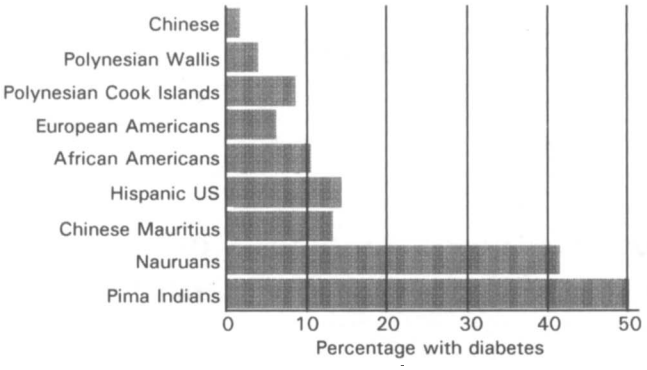

Fig. 1. Age-adjusted prevalence of diabetes in selected populations worldwide. (Adapted from King *et al.* 1993).

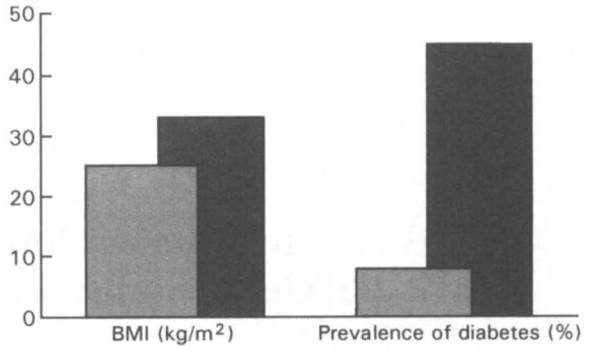

Fig. 3. BMI and prevalence of diabetes among Pima Indians living in Maycoba, Mexico (▨) and Arizona (■). (Adapted from Ravussin *et al.* 1994).

et al. 1995*b*) are additional independent determinants of NIDDM among the Pima Indians. Furthermore, independent of its effects to promote obesity, a high-fat diet is associated with metabolic changes that can worsen glucose tolerance. Compared with a traditional Pima diet (percentage contribution to total energy (% energy): carbohydrate 70, fat 15, protein 15) an isoenergetic 'modern' diet (% energy: carbohydrate 30, fat 50, protein 20) is associated with significantly higher fasting and stimulated plasma glucose concentrations and impaired β-cell function (Swinburn *et al.* 1991). Thus, the adoption of a modern diet higher in fat and lower in complex carbohydrate has undoubtedly played a significant role in the increase in diabetes among the Pima Indians during the last century.

In addition to marked changes in diet, the transition from a traditional to a modern lifestyle was associated also with striking declines in physical activity levels. Decreases in physical activity levels probably contributed to an increase in the prevalence of obesity, but also may have had independent effects in promoting diabetes. A recent survey demonstrated lower lifetime physical activity levels in Pimas with NIDDM than in those without NIDDM, and an inverse relationship between current physical activity and fasting and 2 h glucose levels during oral glucose tolerance testing among non-diabetics (Kriska *et al.* 1993). Although obesity was negatively correlated to physical activity levels in this study, the association between glucose intolerance and low physical activity levels remained significant, even after adjusting for BMI.

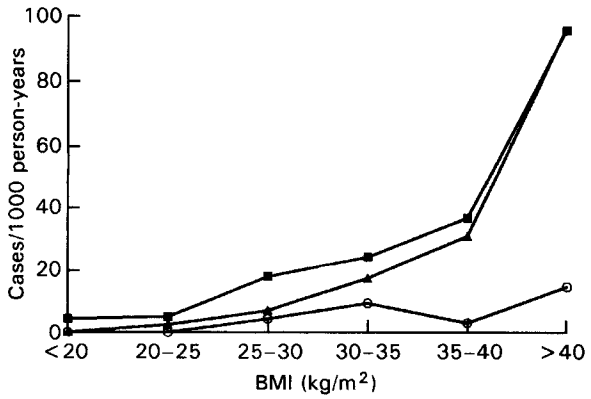

Fig. 2. Age-adjusted incidence of diabetes in 5–44-year-old Pima Indians according to body mass index and parental diabetes status. (■), Both parents with diabetes; (▲), one parent with diabetes; (○), neither parent with diabetes. (Adapted from Knowler *et al.* 1981).

The importance of diet, obesity and physical inactivity in the development of NIDDM in the Pima Indians of Arizona is highlighted by preliminary reports from a survey of Pima Indians living in and around Maycoba, a small village in the Sierra Madre mountains of northern Mexico. This population is believed to have separated from the Pima Indians of Arizona 700–1000 years ago, based on archaeological and linguistic data (Ravussin *et al.* 1994). The rugged mountains that the Mexican Pimas call home have isolated them from Western influences until recently. Even today, the majority of villagers eke out a living by subsistence farming or by working in a local sawmill. The diet of this population resembles a traditional Pima diet, high in complex carbohydrates and low in saturated fats. On average, Pima Indians in Maycoba spend four times as many hours per week in occupational physical activity than do Pima Indians in Arizona. Not surprisingly, obesity is uncommon in this population and the prevalence of diabetes is substantially lower than that among Pima Indians living in Arizona (Fig. 3).

The multi-generational nature of the National Institutes of Health studies of the Pima Indians has made it possible to identify additional environmental or acquired risk factors for developing NIDDM. In particular, the perinatal period appears to be an important time during which exposure to environmental factors can markedly affect the risk of subsequently developing diabetes. For example, hyperglycaemia *in utero* is associated with a substantially higher risk of diabetes. Among 20–24-year-old Pima Indians, 45 % of individuals whose mothers were diabetic during pregnancy were diabetic compared with 1·4 % of individuals whose mothers were non-diabetic during pregnancy (Pettitt *et al.* 1988). This excess risk is largely attributable to an effect of hyperglycaemia on intrauterine development, rather than shared genetic determinants between the mother and child, as diabetes developed in only 8·6 % of 20–24-year-old offspring of mothers who became diabetic subsequent to delivery. Birth weight is another perinatal risk factor for diabetes among Pima Indians, with both low and high birth weights conferring excess risk (McCance *et al.* 1994). The prevalence of diabetes was 30, 17 and 32 % respectively among 20–39-year-olds whose birth weight was low

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Table 1. Environmental factors promoting type 2 diabetes mellitus in Pima Indians

High-fat diet
Obesity
Physical inactivity
Hyperglycaemia *in utero*
Low or high birth weight
Bottle-feeding

(<2500 g), normal (2500–4500 g) and high (>4500 g). Finally, a recent study suggests that events in the immediate postnatal period also affect risk of diabetes. The odds ratio for developing diabetes was 0·41 among infants who were exclusively breast-fed during the first 2 months of life compared with infants who received some bottle-feeding during this period (Pettitt *et al.* 1997).

Clearly, environmental factors, including changes in diet, obesity and physical activity, contributed to increases in the prevalence of NIDDM in the Pima Indians during this century (Table 1). As more women of child-bearing age develop diabetes before or during pregnancy, the prevalence of diabetes increases in successive generations, thus creating a vicious cycle that further amplifies the problem in the community.

What is the evidence that genetic factors contribute to the development of type 2 diabetes mellitus in Pima Indians?

The rapid increases in the prevalence of diabetes during this century and the clear indications that changes in lifestyle contributed to this phenomenon seem incongruent with a significant genetic basis for NIDDM in the Pima Indians. However, other evidence suggests that genetic factors increase susceptibility to NIDDM in this population (Table 2). In general, the observation that the prevalence of diabetes varies widely among diverse ethnic groups sharing a common environment (McCarthy & Hitman, 1993) favours a genetic basis for the disease. The prevalence of NIDDM is approximately 10-fold higher in the Pima Indians of Arizona than in the general US population. Although the Pima Indians have experienced large changes in diet during this century, recent surveys suggest that their current dietary intake is similar to that of the general US population (Smith *et al.* 1996). Furthermore, the increased risk of diabetes among the Pimas is not entirely explained by higher rates of obesity, as the incidence is higher than that in the general population, even among lean Pimas with a BMI $< 24\,kg/m^2$ (Knowler *et al.* 1978, 1981). The familiality of NIDDM is another argument favouring a genetic basis for the disease. As in other populations, diabetes in the Pima Indians is strongly familial (Knowler *et al.* 1981; McCarthy & Hitman, 1993). Diabetes developing before age 45 years increases the risk of diabetes to the offspring 2–4-fold in this population (Fig. 2). Moreover, factors which predict the development of diabetes, including obesity, higher fasting and 2 h glucose and insulin concentrations during an oral glucose tolerance test, insulin resistance and impaired insulin secretion (Knowler *et al.* 1993; Lillioja *et al.* 1993), are themselves

Table 2. Evidence that type 2 diabetes mellitus has a genetic basis in Pima Indians

Higher prevalence compared with general population
Higher risk among first-degree relatives of affected individuals
Familiality of metabolic precursors
Segregation model

highly heritable traits in Pima Indians (Sakul *et al.* 1997). Finally, segregation analyses in this population are consistent with the presence of a major gene affecting age of onset of diabetes (Hanson *et al.* 1995*a*). Collectively these findings suggest NIDDM has a substantial genetic basis in Pima Indians.

Pima Indians as a model population to study the genetics of type 2 diabetes mellitus

The extensive database of phenotypic characterizations and family structure, the tendency for tribal members to stay in or near the community and the fact that Pima Indians are likely to be more genetically homogeneous than Caucasian populations make the Pima Indians an attractive population in which to search for genes for diabetes (Bogardus & Lillioja, 1992). In addition, the heritability of diabetes and its precursors and the presence of a segregation model consistent with a major gene effect enhance the likelihood of finding genes for diabetes in this population (Hanson *et al.* 1995*a*; Sakul *et al.* 1997).

NIDDM is a complex genetic disorder and identification of genes that increase susceptibility to the disease is likely to be a difficult task. With the exception of certain rare forms of the disease, such as maturity-onset diabetes of the young (MODY) (Froguel *et al.* 1993; Yamagata *et al.* 1996*a*,*b*), there is no clear Mendelian pattern of inheritance in most instances. Multiple genes with small to moderate effects are thought to contribute to the development of the disease in the majority of cases. The presence of multiple genes and the potential for interactions between these genes greatly complicates the search for genetic determinants of diabetes. Because the age of onset of diabetes is typically in mid to late life, it can be difficult to collect multi-generational families for classical linkage analyses. Finally, environmental factors strongly influence expression of the disease. Besides these obstacles, which can be found in any population to one extent or another, studying the genetics of diabetes in the Pimas presents some unique challenges. The extraordinarily high prevalence of the disease among the Pimas makes the relative risk to relatives of affected individuals rather low. It also increases the possibility of bilineal inheritance and makes ascertainment of true unaffecteds more difficult. Thus, despite the obvious advantages of this population, there are disadvantages as well (Table 3).

Studies of the genetics of type 2 diabetes mellitus in Pima Indians

Studies of the genetics of NIDDM have progressed rapidly in the last decade as new molecular genetic techniques have

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Table 3. Obstacles to identifying genes causing type 2 diabetes mellitus in Pima Indians

No clear pattern of inheritance
Multiple gene effects
Strong environmental effects
High prevalence
Low relative risk

evolved. Two complementary methods, testing candidate genes and performing a genomic scan with random genetic markers, have been pursued to find genes contributing to NIDDM in the Pima Indians.

In the candidate-gene approach, polymorphisms in genes encoding proteins thought to be involved in some aspect of glucose metabolism are tested for association with diabetes. A large number of genes have been tested in this way, including those encoding insulin, the insulin receptor, insulin receptor substrate-1, glucokinase (*EC* 2.7.1.2), the specific glucose transporters GLUT2 and GLUT4, and glycogen synthase (*EC* 2.4.1.11). In general, these genes are either not linked to diabetes or explain a rather low proportion of the variance. One problem with the candidate-gene approach is that there are hundreds of potential candidates. A detailed knowledge of the pathophysiology of diabetes is helpful in suggesting candidate genes for testing, but it is difficult to determine which physiological, cellular and biochemical abnormalities are primary and which are secondary to the diabetic condition. Another problem with the candidate-gene approach is that causative genes may have no obvious link to glucose metabolism. For example, genes implicated in two forms of MODY (*TCF1* and *TCF14* in MODY3 and MODY1 respectively) encode nuclear transcription factors which, thus, would have been unlikely candidates (Yamagata *et al.* 1996*a*,*b*). Because of these considerations, the candidate-gene approach has been supplemented by performing a genomic scan.

Early linkage studies with genetic markers based on blood group and restriction-fragment-length polymorphisms were limited because these markers were few in number and not very polymorphic. The development of abundant and highly polymorphic polymerase chain reaction-based DNA markers allows the construction of dense genetic maps, which greatly facilitates linkage analyses. This approach has been pursued during the last 3 years in a sample of Pima Indian families selected to be informative for diabetes. Over 1300 individuals from 264 nuclear families have been genotyped at > 500 highly polymorphic random DNA markers distributed on all twenty-two autosomes. Linkage analyses performed using these data suggest a locus at 11q22-23 that is significantly linked to obesity and possibly also to diabetes (Hanson *et al.* 1997).

It is presumed that multiple genes contribute to susceptibility to NIDDM. Since the metabolic precursors of diabetes are themselves highly heritable traits (Sakul *et al.* 1997), we reasoned that genes influencing each of these factors were likely to be fewer in number and, therefore, might be easier to identify than those contributing to the overall syndrome of diabetes. Consequently, in addition to searching for linkage to diabetes, we undertook a genomic scan for linkage to selected quantitative traits predicting diabetes in 363 individuals from 109 nuclear families who had undergone more detailed metabolic studies (Pratley *et al.* 1997). The results of these analyses suggested several loci which may harbour genes contributing to NIDDM in Pima Indians, including 3q21-24 linked to fasting plasma insulin concentration and *in vivo* insulin action, 4p15-q12 linked to fasting plasma insulin concentration, 9q21 linked to 2 h insulin concentration during oral glucose tolerance testing, and 22q12-13 linked to fasting plasma glucose concentration. The evidence for linkage, however, is not strong enough to definitively establish the presence of a diabetogenic gene in any of these regions.

Identifying potential diabetogenic genes in Pima Indians

An approach to identifying genes contributing to NIDDM in Pima Indians is depicted in Fig. 4 and is exemplified by recent studies on *FABP2*, the gene encoding the intestinal fatty acid-binding protein (IFABP). Early analyses suggested a linkage between maximal insulin action, determined during a hyperinsulinaemic–euglycaemic glucose clamp, and a locus (*GYPA/B*) on chromosome 4q determining the MNSs blood group. Polymorphic DNA markers subsequently confirmed the linkage, but suggested that the peak was localized centromeric to *GYPA/B* in a region containing *FABP2* (Prochazka *et al.* 1993). Single-stranded conformational polymorphism analysis and direct sequencing of *FABP2* revealed two silent single nucleotide substitutions and a single nucleotide substitution (guanine to adenine in codon 54 of exon 2) resulting in an amino acid change from alanine to threonine in the protein (Baier *et al.* 1995). The frequencies of the alanine-encoding (Ala54) and threonine-encoding (Thr54) alleles were found to be 0·70 and 0·30 respectively in more than 1000 Pima Indians. In this group, individuals with the Thr54 allele had higher insulin levels during oral glucose tolerance testing and mixed meals compared with those who were homozygous for the Ala54 allele, suggesting that the Thr54 allele is associated with insulin resistance.

Human IFABP is a 15 kD intracellular protein expressed exclusively in the columnar absorptive epithelial cells of the small intestine. This protein contains a single ligand-binding site that non-covalently binds both saturated and

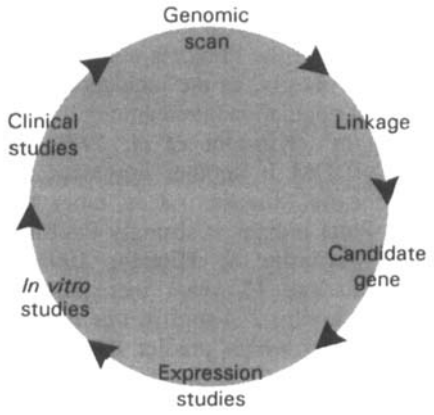

Fig. 4. Approach to finding genes contributing to type 2 diabetes mellitus in Pima Indians.

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unsaturated long-chain fatty acids with high affinity. Since the Ala54 to Thr54 substitution occurs in a region of the molecule involved in fatty acid binding, the binding characteristics of the two forms of the protein were examined *in vitro* using titration microcalorimetry. These studies demonstrated that the Thr54 form of IFABP has a 2-fold higher affinity for long-chain fatty acids (oleate and arachidonate) than the Ala54 form (Baier *et al.* 1995). Furthermore, when expressed in Caco-2 cells, the Thr54 form of IFABP transported long-chain fatty acids and secreted triacylglycerols to a greater degree than the Ala54 form (Baier *et al.* 1996). The increased binding affinity of the Thr54 allele may alter the kinetics of free fatty acid and triacylglycerol appearance into plasma, resulting in delayed and prolonged increases in free fatty acids, triacylglycerols, and lipid oxidation following a meal. This could explain the higher fasting free fatty acid levels and rates of lipid oxidation observed in individuals with the Thr54 allele. It is possible that individuals with the Thr54 allele develop insulin resistance in response to an increase in fatty acid flux through a competitive glucose–free fatty acid cycle and/or by inhibiting non-oxidative disposal of glucose as glycogen. In a subset of 137 genotyped individuals, lower rates of insulin-stimulated glucose disposal were evident in individuals with the Thr54 allele (Baier *et al.* 1995). Studies are currently in progress to test whether the *in vitro* differences in long-chain fatty acid binding affinity and transport between the Thr54 and Ala54 forms of IFABP alter the kinetics of fatty acid absorption *in vivo*.

Gene–environment interactions in the pathogenesis of type 2 diabetes mellitus

The results of studies among the Pima Indians emphasize the importance of both environmental and genetic factors in the pathogenesis of NIDDM. Certain genes apparently increase susceptibility to diabetes, but are not, by themselves, sufficient to produce the disease. Hence, diabetes was virtually unknown among the Pima Indians of Arizona before this century and is still uncommon among the Mexican Pima Indians, who presumably share a common set of genes with the Pima Indians of Arizona, but who retain a traditional lifestyle. Genes increasing susceptibility to diabetes may compromise insulin action and/or insulin secretion, but clinical disease may not be evident unless a certain critical threshold is exceeded. Environmental factors such as a high-fat diet and physical inactivity also may directly compromise insulin action and insulin secretion which, in individuals with susceptibility genes, may result in diabetes. Environmental factors also may increase risk of diabetes by acting in concert with other genes. For example, obesity has a substantial genetic component. The effects of genes increasing susceptibility to obesity are only evident, however, in a modern environment with an abundance of food.

Failure to consider environmental factors may confound the results of genetic analyses of complex disease such as NIDDM. It is possible to account for environmental effects statistically by adjusting for factors known to affect the phenotype to which linkage is being sought. For example, linkage analyses with quantitative traits predicting diabetes (e.g. insulin action) were performed after adjusting for age, sex, obesity and body fat distribution (Pratley *et al.* 1997). This approach has the advantage of simplicity, but because it assumes that the effect of the covariate is not genetically mediated, the power to detect linkage may be reduced in some cases if a certain gene affected both obesity and insulin action. Adjusting insulin action for obesity before performing linkage analyses may obscure the pleiotropic effect of such a gene. Newer computational algorithms allow covariates to be included directly into analyses and may result in increased power to detect linkage in circumstances such as these (Amos, 1994). Unfortunately, most studies of NIDDM have not collected the phenotypic data that would allow for extensive analyses of this type.

The pattern of expression of an individual's genetic complement changes from developmental stage to developmental stage according to a well-defined cycle. It is also evident from studies among the Pima Indians that gene–environment interactions are not static, but instead are dynamic, evolving throughout an individual's lifespan. Exposure to certain environmental factors may be important at certain critical times of life; for example, *in utero*, hyperglycaemia is evidently capable of affecting some aspect of fetal development in a way that increases the risk of subsequently developing diabetes. Likewise, bottle-feeding during the first 2 months of life also may affect development and increase risk. Ageing may further compromise insulin action and/or insulin secretion which, when combined with other genetic or acquired factors, leads to diabetes. A theoretical scheme in which multiple insults during critical developmental stages eventually lead to NIDDM is depicted in Fig. 5.

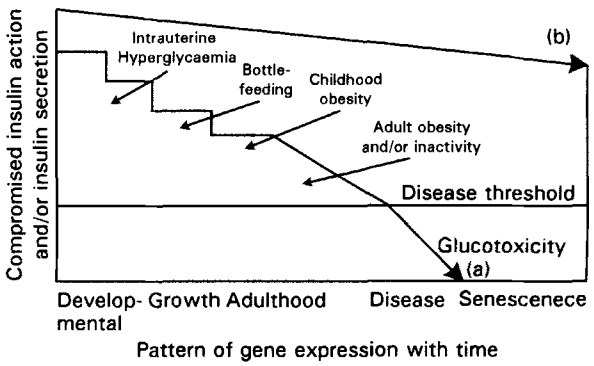

Fig. 5. Theoretical scheme depicting the progression to diabetes in two individuals. The first ($\xrightarrow{(a)}$) starts life with certain susceptibility genes which impair insulin action and/or insulin secretion. Hyperglycaemia *in utero* alters prenatal development in such as way as to further compromise insulin action and secretion. Similarly, bottle-feeding in the immediate postnatal developmental period impairs metabolic performance. A high-fat diet, physical inactivity and the development of obesity in childhood and the adult years additionally compromise insulin action and/or insulin secretion. Eventually the threshold of clinical disease is crossed. Glucotoxicity may further compromise insulin action and secretion once clinical disease is evident. In contrast, the second individual ($\xrightarrow{(b)}$) is born without susceptibility genes, exercises and eats a healthy diet and does not become obese. Insulin action and/or insulin secretion are compromised only slightly, if at all, by the ageing process and this individual never crosses the threshold determining clinical disease.

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Summary

The comprehensive longitudinal studies of diabetes conducted in the Pima Indians of Arizona over the last 30 years indicate that both genetic and environmental factors play a critical role in the pathogenesis of the disease. Pre- and postnatal exposures as well as diet and physical activity in adulthood markedly affect risk of developing NIDDM in this population. In addition, the high prevalence of diabetes in the Pimas relative to other populations and the familiality of the disease and its precursors, strongly suggest a substantial genetic basis. Interactions between genes and the environment are obviously important in the pathogenesis of NIDDM, but it remains unclear exactly how these interactions occur and how to adequately account for these effects when searching for genes contributing to diabetes. The realization that gene–environment interactions are significant, and may be the dominant mechanism increasing susceptibility to NIDDM, should encourage further investigations. Future progress in studying the genetics of NIDDM and other complex diseases will come not only from technical advances currently in development, but also from advances in understanding the pathophysiology of the disease and the role of gene–environment interactions, and a renewed emphasis on careful clinical characterization of subjects participating in these studies.

Acknowledgements

The author gratefully acknowledges the cooperation and support of the leaders and people of the Gila River Indian Community and invaluable contributions to advancing our understanding of the pathogenesis of diabetes this population has made over three decades. The success of these studies would not have been possible without the careful collection and analysis of data by the many scientists, health care providers and technical staff affiliated with the project over many years. Finally, the author is indebted to Drs Peter H. Bennett, Clifton Bogardus and William C. Knowler for their leadership and invaluable comments.

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https://doi.org/10.1079/PNS19980029 Published online by Cambridge University Press