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Letters to the Editor

Hyperproinsulinaemia in cirrhosis

Dear Sir

Taylor and Alberti, confirming our finding of hyperproinsulinaemia in patients with cirrhosis, at the same time questioned the correctness of the selection of the group of healthy subjects serving as controls in this study [1, 2]. In particular, they stressed the difference between levels of blood glucose after oral loads of 50 and 100 g glucose and the prolonged maintenance of raised serum immunoreactive insulin (IRI) and C-peptide levels after 100 g glucose in these subjects. Their remarks require a response.

Our study was begun before the WHO proposal of modifying the oral glucose tolerance test to a 75-g glucose load and at that time a 50-g load served as the basis for the selection of subjects, as was described in detail in our study. The oral 100-g glucose load was used for stimulation of the secretion of insulin, C-peptide and proinsulin in order to obtain results comparable with those of other investigators, who generally used this dose of glucose.

The problem of blood glucose curve patterns in healthy subjects after ingestion of 50 and 100 g glucose has not been clarified so unequivocally as suggested by Taylor and Alberti. In a study of 50 healthy subjects, a significant difference in the course of blood glucose curves was found between 90 and 180 min of oral glucose loading [3] and in another study a similarly significant difference was observed as early as 30–120 min following oral administration of 50 and 100 g glucose [4].

In healthy subjects evident differences are observed in IRI and C-peptide (and proinsulin) concentrations in serum following oral loads of 50 and 100 g glucose. In tests prolonged to 300 mins it could be shown that after 50 g glucose – in agreement with the remarks of Taylor and Alberti – the maximal increases in serum IRI and C-peptide occurred between 45 and 60 min and the levels of these peptides fell rapidly after that time. On the other hand, after a load of 100 g glucose the rise in serum IRI levels persisted as a plateau or as a double peak between 30 and 120 min, and returned to the initial value only between 240 and 300 min. A similar shape was observed in the serum C-peptide curve in these subjects [3]. This finding shows that the prolongation of raised IRI and C-peptide levels observed in our control subjects after an oral load of 100 g glucose is a phenomenon which is normally found in healthy subjects.

Although two of our healthy subjects had borderline blood glucose levels (exceeding the upper normal limit) 60 min after a 50-g oral glucose tolerance test, we do not think this of real significance for the interpretation of our results. Discussion of the definition of health in relation to carbohydrate metabolism is obviously very difficult, since at this juncture we enter a field where from time-to-time borderlines are arbitrarily changed and frequently questioned:

"Grammatici certant et adhuc sub iudice lis est"

(Horatius: Ars Poetica, line 78)

Yours sincerely,

T. Kasperska-Czyzykowa, L. G. Heding and A. Czyzyk

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Diet and insulin-dependent diabetes in the BB rat

Sir,

We would like to comment on the Short Communication from Elliott and Martin [1]. The authors fed diabetes-prone BB rats four diets from weaning as follows: chow (control); group 1: semi-synthetic diet in which protein was replaced with l-amino acids; group 2: contained 1% gliadin in addition to the aforementioned; and group 3: contained 1% skim milk powder instead of gliadin. The incidence of diabetes was 19/39, 3/19, 7/20 and 11/21, respectively. From these results they concluded, "Accordingly, the presence of intact protein appears necessary for the full expression of the genetic susceptibility to develop diabetes in this colony of BB rats".

The finding that animals fed skim milk powder, in addition to the base diet, developed diabetes in numbers comparable to heavier chow-fed rats is interesting but the authors' conclusion that the effect was due solely to protein is clearly open to debate since skim milk powder contains many other constituents besides protein as shown in their Table 1 [1]. Furthermore, since the chow "control" diet was not isocaloric with respect to the test diets, intake of *all* nutrients in chow-fed rats would differ from that of animals on the test diets.

Since there is probably an interaction of environment and genetic background in development of the syndrome [2, 3], the authors should have distributed littermates equally among all groups, including the chow control group. Were there litter effects – how many of the seven litters in group 3 produced diabetic rats? How many litters were in the chow-fed control group?

The use of a closed formula diet, such as chow, as the sole control was unfortunate since this mixture of chemicals is not only ill-defined but varies with changes in the market place. It is preferable to use standard, purified diets, such as the AIN-76 diet, which consist of commercially refined protein, carbohydrates, fat and defined mineral and vitamin mixtures [4]. The diet constituents reported by Elliott and Martin were described only as "carbohydrates, fat, salt mixture and vitamins", etc. What was the source of carbohydrate and fat and which salt and vitamin mixtures were used? The "control" chow diet contained 50.0% carbohydrate, 5.0% fat and 5.2% fibre, compared with 64.2% carbohydrate, 10.0% fat and no fibre in the "semi-synthetic base". The 5.2% fibre reported in the "control" chow diet must have been crude fibre; the actual dietary fibre content of this diet was probably 15%. Whether or not these major differences can account for the more than 40% greater body weight of chow-fed rats is difficult to determine without more detailed diet information and data on food and water intake. It is clear that other purified diets, such as AIN-76, give equal or even better growth rates compared with chow [4], strongly suggesting that the semi-synthetic base diet used by Elliott and Martin was nutritionally inadequate. To date we have fed more than 300 BB rats using modified AIN-76 diets with growth rates equal to or better

than those of chow-fed animals. How does one interpret the effects of varying amino-acid sources when they are superimposed on a background of inadequate nutrition? It seems not to have occurred to the authors that expected incidence values, which they calculate using the incidence in their (presumably) chow-fed colony, relate to animals on chow only. To put the question differently, what was it about diet 1 (the semi-synthetic base diet) that protected the animals from becoming diabetic? It is difficult even to speculate since we are not told what was in the diet.

It is possible that chow contains diabetogens or co-diabetogens, or lacks immune system modifiers which protect animals from developing diabetes and/or insulitis. Indeed, we have raised these questions as a result of previous studies comparing a chow diet with a modified AIN-76 purified diet [5, 6]. We are presently investigating the possibility that diabetogens may be present in some batches of laboratory rat chow and are continuing to examine the capacity of various nutrients to modify expression of the syndrome through changes in the immune system. It is conceivable that variations in diet may explain differences in incidence of diabetes in BB rat colonies from various locations. Such chow-derived differences may have been the reason that one earlier study failed to detect diet-related changes in expression of the syndrome [7].

In studies of this kind it is important to attempt to determine whether or not one has merely delayed onset of the overt syndrome or has actually prevented its expression. There are two points which the authors should consider here with regard to this work: (1) 133 days is probably not long enough to differentiate delay from prevention, (2) from the literature [8] and our own experience, BB rats present as overtly diabetic and asymptomatic/diabetes-prone. The latter category must be further divided into those with or without some degree of islet inflammation. Since the authors have chosen in the current work to consider their animals as either diabetic or non-diabetic, it is impossible to discern the true effects of the diets, i.e., how many of the 16 "non-diabetics" on the group 1 diet showed signs of insulitis? The authors claim all non-diabetics were "normal". In our experience this is highly unlikely and leads one to question whether the authors used step and/or serial sectioning to examine the pancreas. This approach has been taken by us as well as others [8].

Given the large number of chemicals in any diet and the difficulty in modifying one constituent without affecting others, the question of an effect of diet on the BB diabetic syndrome or any other disease process must be approached with as much rigour as we can feasibly muster. Although we are pleased to see support for our earlier findings that diet can alter diabetes incidence in the BB rat [5, 6, 9–10], the lack of essential information on dietary constituents and on signs of insulitis in the asymptomatic/diabetes-prone animals in the Elliott and Martin experiment raises questions that must be considered when interpreting their results.

Yours sincerely,

F. W. Scott, R. Mongeau and W. A. Behrens

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Reply from the authors

Dear Sir,

Our study on the effect of dietary natural proteins on the development of diabetes in the BB rat [1] was prompted by the clinical observations of Dr. R. B. Elliott on Polynesian children living in Western Samoa and following their migration to New Zealand. At that time, the only publications on possible dietary triggers of insulin-dependent diabetes were (1) the observation of Helgason and Jonasson on the effect of smoked meat eaten by mothers during the Christmas festivities in Iceland [2] and (2) the negative observation of Rossini et al. [3] in which they found no effect on the development of diabetes in BB rats fed regular Purina Chow with addition of carbohydrates, fats or proteins. The results of our initial experiment seemed to provide original and potentially important information suitable for a brief communication. We took special care to avoid undue speculations and to provide a strict factual description of our findings. Dr. F. W. Scott and his colleagues complain of the lack of information about dietary compositions. Within the limits of a Short Communication we included the essential data plus information about the suppliers, since all are commercially available.

Their main criticism refers to the control group fed chow. The inclusion of this group was intended solely as a monitor of the usual incidence of diabetes in the colony. It consisted of eight litters which were delivered immediately before or after the delivery of those litters included in the experimental group. Our hybrid BB colony, maintained under germ-free conditions for the last 4 years, has a remarkably constant mean incidence rate of diabetes of approximately 50%. (The lowest recorded incidence was 36%).

The statement "that the semi-synthetic base diet was nutritionally inadequate" is questionable. It is known that such diets do not promote maximum weight gain in rats but they efficiently support growth during the most active post-weaning period [4] as well as during reproduction and lactation [5, 6].

We looked for, but did not observe, any litter effect in the various groups, including group 3.

It is difficult to understand what Dr. Scott et al. consider as a 'normal' islet. In our report we identified as 'normal' those islets with fully granulated β cells without *any* mononuclear infiltration. Although not reported in the paper, the pancreatic insulin content was grossly different between diabetic and non-diabetic rats (9.48 ± 2.2 versus 119.6 ± 37.0 ng/mg wet tissue, respectively, mean $\pm$ SD).

Drs. Scott, Mongeau and Behrens seem to be unhappy with our observations which they contrast with a number of speculations of

their own. Unfortunately it is not possible to comment on their work at this time since this has not been published except for two experiments reported in four abstracts. What is clear from those abstracts is that the authors had also chosen to consider their animals as either diabetic or non-diabetic without reference to the asymptomatically diabetic rats.

Yours sincerely,

J. M. Martin and R. B. Elliott

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Insulin binding and internalization in human and chicken erythrocytes and the role of the cell nucleus

Dear Sir,

Nerurkar and Gambhir [1] have critizised a recent publication from this laboratory [2], based on their biochemical studies of insulin binding. Our studies reported our experiences with electron microscopy on the same topic. In these studies, we showed that insulin-gold complexes were internalized only by erythrocytes still displaying a nucleus (chicken, frog and trout) and also by human reticulocytes which had remnants of nuclear material. Mature erythrocytes did not internalize insulin-gold complexes.

The contradictory opinions about the internalization of insulin by mature human erythrocytes arise from two different methodological approaches. We must state, however, that in preparation for electron microscopy, we did carry out binding studies with ^{125}I-insulin, both at 15 °C and 37 °C with human and chicken erythrocytes. After an incubation time of 120 min, the cells were washed three times with an acidic incubation buffer (pH 6.0) to remove the non-covalent-bound hormone from the cell surface [3]. The remaining radioactivity in the cell pellets is shown in Table 1. Chicken erythrocytes revealed an almost ninefold greater percentage of covalently bound ^{125}I-insulin, which could be regarded as the internalized fraction. While the biochemical approach alone did not satisfactorily solve the question of whether insulin had or had not been internalized, the results of the complementary electron microscopy studies confirmed our hypothesis that insulin-gold complexes can be internalized only by nucleated erythrocytes and by human reticulocytes. On the other hand, in mature human erythrocytes, insulin-gold complexes were found only on the cell surface and not within the cell. Ackerman and Wolken [4] found a similar result using gold-labelled insulin albumin.

According to strict morphological criteria, reticulocytes are non-nucleated cells, although they can carry out synthesis of membrane proteins [5], a process which seems to be insulin-dependent and not seen in the mature erythrocyte [6]. Ginsberg and Brown [7] related the inability of mature erythrocytes to synthesize protein to the fact that these cells do not down-regulate their insulin receptors. Our referral to reticulocytes as nucleated cells was thus meant in a metabolic sense (= protein synthesis).

Table 1. The remaining radioactivity in the cell pellets of human and chicken erythrocytes before and after three washes with incubation buffer

Erythrocytes	%-Specific ^{125}I-insulin bound to erythrocytes (4×109 cells/ml)	
	Initial binding	Binding after three washes with acidic buffer (pH 6.0)
Human ($n = 6$)	7.6 ± 1.6[a]	0.62
Chicken ($n = 6$)	13.7 ± 2.9	5.22

Results expressed as mean ± SEM. [a] Reticulocyte count in the isolated fractions < 4.3 per 1000

Ivarsson and Thorell [8] have documented that insulin binding to erythrocytes is a function of the percentage of reticulocytes in the isolated cell fraction. We have shown that reticulocytes can internalize insulin. The studies of Nerurkar and Gambhir [1, 9] showed that erythrocyte lysates can degrade ^{125}I-insulin. However, they have failed to determine the percentage of reticulocytes present in the isolated cell fraction. We assume that the degradation activity described by Nerurkar and Gambhir could well arise from a metabolically active part of the erythrocyte fraction, namely the reticulocytes.

Yours sincerely

Ch. Thun, X. Bihr, R. Moncayo and E. F. Pfeiffer

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