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GLUCAGON TREATMENT IN NICTH

To the editor:

In their recent case report (*JCEM*, September 1995) R.C. Baxter *et al.* (1) described the therapeutic efficacy of glucocorticoids and growth hormone (GH) for an elderly woman with nonislet cell tumor hypoglycemia (NICTH). In addition to the elegant delineation of complex dysregulation of insulin-like growth factor (IGF) homeostasis, the report points to the practical difficulties of prolonged GH administration in such patients. In addition, long-term glucocorticoid treatment is not without hazard, even though in this patient modest prednisone dosage proved a useful alternative.

Recalling that, in patients with NICTH, glucagon infusion causes an acute increase of glucose levels (2), we have had the opportunity to treat such patients with continuous intravenous infusion of glucagon and to achieve durable correction of hypoglycemia. In our previously reported case (3) the dose required was 0.3 mg/h infused via Infumed pump (Medfusion, Inc, Norcross, GA). In a subsequent patient (unpublished) lesser doses were needed; continuous infusion of dextrose can also be used with good results.

Prolonged survival is now possible for many patients with malignancies associated with NICTH, and adequate control of hypoglycemia becomes an important component of successful therapy. We suggest that long-term infusion of glucagon and even dextrose can be added to glucocorticoids and GH as effective treatment alternatives.

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PATHOGENESIS OF MYXEDEMATOUS ENDEMIC CRETINISM

To the editor:

We refer to the study by Chiovato and coauthors (1), in which it is claimed that humoral thyroid autoimmunity is not involved in the pathogenesis of the atropic (not athyreotic as the authors mentioned in the introduction) thyroid gland frequently found in myxedematous endemic cretinism. We disagree with the interpretation given by the authors of present and past data for a number of reasons:

1. Chiovato *et al.* (1) studied two different populations with obvious ethnical differences (Peruvian Indians and Italian caucasoids). The subjects living in the Andean region are descendants of the indigenous inhabitants of the Andes, dominated by the Incas at the beginning of the 15th Century. Both in Ecuador and Peru, they share the same living conditions, habits, socioeconomic background, and a common language (Quechua). What is remarkable about these Indians is that autoimmune thyroid disease is virtually unknown among this population. On the other hand, subacute thyroiditis seems to be very frequent and is seasonal in character (2). The only few confirmed cases of Hashimoto's thyroiditis in Quito (Ecuador) were detected in subjects of European extraction. More recently, Fierro-Benitez (1995 personal communication) conducted a survey in the Tocachi village (Ecuador) using ultrasonography, fine needle biopsy, and a sensitive assay for serum antiperoxidase antibodies. Although more than 80% of the population was evaluated there was not a single case of chronic autoimmune thyroiditis. It is tempting to conclude that it would be difficult to compare the data obtained by Chiovato et al. (1) in endemic cretins from this Peruvian population to that of other ethnical groups with different genetic background.
2. Another point is the age of the patients with endemic cretinism. We have previously demonstrated that the older the neurologic endemic cretin, the more frequently he or she will develop hypothyroidism later in life (3). Probably this is the result of progressive deterioration of the thyroid gland caused by a number of local and environmental agents. In the case of thyroid atrophy detected early in childhood and in adolescence, it is conceivable to imagine the presence of an autoimmune mechanism. However, once the thyroid gland is destroyed the serum levels of antibodies to thyroid antigens tend to disappear with time. In the paper by Chiovato *et al.* (1), the Italian cohort of endemic cretins ranged in age from 32–72 yr. Thus, it is possible that blocking antibodies were not found because of the lack of thyroid antigens capable of inducing an autoimmune response in this older group of endemic cretins.
   Moreover, looking carefully at Fig. 4 of the paper by Chiovato *et al.* (1) it is shown that IgG from three out of six endemic cretins with thyroid atrophy inhibited TSH-stimulated growth by an index of between 16 and 30%. Going back to our own studies (4, 5), it is shown that 2 mg IgG from endemic cretins inhibited less than 20% TSH-stimulated growth in three subjects, about 40% of TSH-stimulated growth in two subjects and more than 75% of TSH-stimulated growth in two individuals. Therefore, only four IgGs of the seven endemic cretins had a significant inhibitory effect on TSH-stimulation growth. This is

comparable to the results obtained by Chiovato *et al.* (1). It should be mentioned that these four patients were adolescents (15–16 yr) or young adults (30–33 yr), as opposes to the older group studied by Chiovato *et al.* (1).

3. Last but not least remains the question of the methodology employed by Chiovato *et al.* (1) and Boyages *et al.* (6).
   The thyroid growth blocking IgGs were determined in endemic cretins in China (6) by measuring DNA synthesis in guinea pig thyroid explants by means of nucleic acid (Feulgen) cytophotometry. This test detected at least 100-fold smaller amounts of TSH than do conventional assays. Results of DNA synthesis obtained in the cytochemical bioassay correlate well with the mitotic index in a cell culture system. Therefore the argument by Chiovato *et al.* (1), that the incubation time with TSH is shorter than would be necessary for DNA synthesis, seems to be irrelevant.
   In the Chinese endemic cretins preparation of IgG and subsequent purifications was performed with a column containing protein A Sepharose and subsequent dialysis (6), while Chiovato *et al.* (1) separated IgG into Sephadex columns with subsequent precipitation by ammonium sulphate. As commented by Brown (7), a significant decrease in TSH-binding inhibitor may occur with the latter preparation. Finally, the presence of the thyroid-growth blocking autoantibodies was positively related to thyroid atrophy (shown by ultrasound) and serum TSH levels in the Chinese endemic cretins, whereas no attempt was made by Chiovato *et al.* (1) to assess the thyroid volume by sonography or to correlate their findings to serum TSH concentrations.
4. It is worth mentioning at this point that DeQuervain and Wegelin (8) had found lymphocytic infiltrates (as found in autoimmune thyroiditis) in some of these atrophic glands of endemic cretins. This could be considered pathological evidence for an autoimmune role in the atrophic process of the thyroid gland.

To conclude, until new evidence is provided one is left with the same assertion posed by Brown (7): Immunoglobulins afffecting thyroid growth will be a continuing controversy.

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PATHOGENESIS OF MYXEDEMATOUS ENDEMIC CRETINISM—AUTHOR'S RESPONSE

To the editor:

TSH receptor autoantibodies that compete with TSH for its receptor and block TSH-stimulated thyroid cell function and growth (TSHBAb) have been found by us (1) and others (2, 3) in patients with autoimmune thyroiditis. In virtually all these patients TSHBAb are associated with other thyroid autoantibodies such as thyroglobulin (TGAb) and thyroperoxidase (TPOAb) antibodies (1, 3). The prevalence and levels of TSH-receptor antibodies able to block thyroid function and growth were found to be inversely correlated with the size of goiter and the degree of thyroid failure (1). TSHBAb, TGAb, and TPOAb were also found in rare neonates with sporadic congenital hypothyroidism born to mothers with active thyroid autoimmune disorders (4). In our study on endemic cretinism in Italy and Peru (5), none of the immunoglobulin G (IgG) from 39 cretins was able to inhibit TSH-stimulated thyroid cell function or growth. The frequency of TGAb and TPOAb in endemic cretins was relatively low and did not differ from that observed in normal subjects living in the same endemic area. Our results do not confirm the studies of Boyages (6) and Tsuboi (7), in which antibodies able to block TSH-stimulated thyroid cell growth (ThG-blocking Ab) were apparently found in the great majority of nongoitrous hypothyroid endemic cretins from China (6) and Brazil (7). In all Chinese and Brazilian endemic cretins TGAb, TPOAb, and TSH-receptor antibodies by radioreceptor assay were undetectable, while inhibition of TSH-stimulated cAMP was not tested. No explanation was offered for the unusual finding that in endemic cretins only one putative thyroid antibody was positive, while all the other commonly observed thyroid antibodies were negative. As previously indicated, our study in endemic cretins from Italy and Peru did not confirm these unusual results (5). In their letter in this issue, Boyages and Medeiros-Neto raise the following criticisms to our paper:

1. Peruvian cretins were recruited in a genetically segregated or restricted population devoid of thyroid autoimmunity;
2. Endemic cretins in our study were older than those studied in Brazil;
3. The methodology used in our study was inadequate for the detection of ThG-blocking Ab;
4. There are pathological observations suggesting an autoimmune pathogenesis of endemic cretinism.

To answer these criticisms, we offer the following arguments:

1. *Genetic restriction of Peruvian cretins*

Peruvian cretins included in our study cannot be considered a homogeneous group of Quechua descendants. Most of them have Spanish names, and many are whites. Indeed, most Peruvian people in the Andean mountains are mestizos, and in Northern Peru, where cretins included in our study were recruited, the influence of indigenous race is low. As a matter of fact, Peruvian cretins in our study did not come from a genetically restricted or homogeneous population, as that studied by Fierro-Benitez in the Tocachi village in Equador. The statement that "autoimmune thyroid disease is virtually unknown" is perhaps applicable to genetically restricted people living in some Andean villages, but not to the Indian population living in the Andes as a whole. Evidence for this includes the following: 1) thyroid autoantibodies can be found in 14% of Andean pregnant women (8); 2) 25% of Graves' patients attending the Cayetano Heredia University Hospital in Lima come from Andes (9); 3) chronic thyroiditis diagnosed by fine needle aspiration biopsy was reported in 14% of patients living in endemic areas in Peru (10); 4) a recent study performed in La Paz (Bolivia) has shown a high prevalence of thyroid autoantibodies in Andean Indians, European descendants, and mestizos referred to a National Institution for hyperthyroidism and hypothyroidism (11).

A further argument against the criticism that genetic restriction was responsible for negative results in our study is the fact that we failed to detect ThG-blocking Ab in Italian cretins, coming from a mixed Caucasian population (5).

2. *Age of cretins*

A second criticism claims that endemic cretins in our study were older than in previous reports (6, 7) and raises the possibility that this difference could explain our negative results because of disappearance of ThG-blocking Ab with increasing age. Several arguments contradict this criticism.

The group of 29 Peruvian cretins in our study (age range: 9–60 yr) included children and young adults (18 patients were younger than 30 yr). Thus, their age range was comparable to that of cretins investigated in the paper of Tsuboi *et al.* (7). We agree that the Italian cohort of endemic cretins was older than that studied in Brazil (5), but in the Chinese population investigated by Boyages *et al.* (6), 9 out of 14 myx-

edematous endemic cretins were older than 30 yr, and still all but one were positive for ThG-blocking Ab.

The hypothesis that in hypothyroid nongoitrous endemic cretins ThG-blocking Ab may disappear with increasing age is also unlikely. While hypothyroidism persists throughout adult life in endemic cretins, in those documented cases of sporadic autoimmune hypothyroidism in which TSHBAb alone is the cause of thyroid failure, recovery of thyroid function occurs upon disappearance of the blocking antibody. This phenomenon has been clearly described in neonates born to mothers with autoimmune thyroid disease (4) and in some cases of adult autoimmune hypothyroidism caused by TSHBAb (12). On the other hand, TSHBAb and other thyroid antibodies (TGAb and TPOAb) persist for many years after diagnosis in most patients with permanent autoimmune hypothyroidism (1, 3, 12). As indicated above, it is thus surprising that in previous studies (6, 7) none of myxedematous endemic cretins who were positive for ThG-blocking Ab had other thyroid antibodies in their serum.

With specific regard to the possibility that thyroid antibodies disappeared from serum in older endemic cretins, it may be relevant to recall that a major cause of thyroid antibody reduction in patients with autoimmune hypothyroidism is the institution of replacement therapy with L-thyroxine (13). This was not the case in our study, since all samples from endemic cretins were obtained before initiation of treatment.

We also disagree with the interpretation proposed for the results in Fig. 4 of our paper (5), and on their alleged similarity to those of Tsuboi *et al* (7). In view of the following discussion it is relevant to recall that both studies used the FRTL-5 cell system to detect ThG-blocking Ab. However, different experimental conditions and, in particular, a different dose of TSH were employed.

As shown in Fig. 4 of our paper (5), the TSH-stimulated growth inhibition index ranged from −30 and +35%, with IgGs from normal subjects living in iodine-deficient or iodine-sufficient areas. From these findings we concluded that results of up to 35% inhibition index could not be considered positive for ThG-blocking Ab. As a matter of fact, no IgG from nongoitrous hypothyroid endemic cretins showed an inhibition index greater than 30%, and the results obtained were superimposable to those produced by IgGs from euthyroid or hypothyroid endemic cretins with goiter. Similar results were obtained by measuring DNA accumulation in FRTL-5 cell cultures. When enough sample was available, increasing amounts (0.1–2 g/L) of IgGs from nongoitrous hypothyroid endemic cretins were added to the system, but no dose-dependent inhibition was observed. In contrast, IgGs from selected patients with autoimmune atrophic thyroiditis produced a strong and dose-dependent inhibition of TSH-stimulated ^{3}H-thymidine (^{3}HTDR) incorporation and DNA accumulation. The same IgGs were potent inhibitors of TSH-stimulated cAMP accumulation in FRTL-5 cells. The above findings are in sharp contrast to those of Tsuboi *et al.* (7). In the latter study, 4 out of 7 IgGs from nongoitrous hypothyroid endemic cretins produced an inhibition of TSH-induced ^{3}HTDR incorporation greater than 50%. Two of these IgGs inhibited the effect of TSH by more than 75%. These impressive inhibitory results cannot be ascribed to a greater sensitivity of the method employed by Tsuboi *et al.* (7). In our system for the detection of ThG-blocking Ab, IgGs were challenged against a much lower concentration of TSH than that used by Tsuboi *et al.* (30 mU/L *vs.* 100 mU/L). As clearly demonstrated in a previous study from our laboratory (1), this dose of TSH induces a reproducible increase of cAMP and ^{3}HTDR incorporation in FRTL-5 cells and allows a higher sensitivity in the assay for antibodies blocking TSH effect with respect to 100 mU/L of the hormone.

In view of the above discussion, the correct interpretation of our results is that, at variance with Tsuboi *et al.* (7), we did not detect ThG-blocking Ab in IgGs from hypothyroid nongoitrous endemic cretins.

3. Methodology

The third criticism regards the methodology used in our study. The point is made that the Feulgen cytochemical bioassay (CBA), being supersensitive, could detect ThG-blocking Ab at a much lower concentration than any other currently available method. Indeed, it is reported that the Feulgen CBA can measure the effect of TSH at a much lower concentration than 1 mU/L (14). In particular, in the report of ThG-blocking Ab in hypothyroid endemic cretins, TSH was used at a concentration of 0.1 mU/L to stimulate growth (6).

In our FRTL-5 cell system (5), TSH produced a significant stimulation of ^{3}HTDR incorporation, and DNA accumulation at concentrations comprised between 1 and 10 mU/L. These concentrations of TSH are very close to the levels of TSH present in human serum under physiological conditions or in the initial phase of hypothyroidism. In the same culture conditions it is possible to show a parallel increase of cAMP production, ^{3}HTDR incorporation, and DNA accumulation induced by TSH (15). Thus, the same range of TSH concentrations stimulates both cAMP accumulation and cell growth in FRTL-5 cells. These experiments provide a validation of our growth assay, as the adenylate cyclase/cAMP cascade is considered the main pathway involved in the growth of FRTL-5 and human thyroid cells (16). In our system a significant inhibition of ^{3}HTDR incorporation was elicited by IgGs from patients with autoimmune atrophic thyroiditis that also inhibited TSH-stimulated cAMP accumulation (5). This effect was still evident when IgGs were diluted to a concentration (0.1 g/L) similar to that found to be effective in the Feulgen CBA for ThG-blocking Ab (6). We therefore assume that, in the working conditions used for the detection of ThG-blocking Ab, our system is not less sensitive than the Feulgen CBA.

On the other hand, the Feulgen CBA used in the study of Boyages (6) to measure ThG-blocking Ab has been widely criticized by Dumont *et al.* (16) and Zakarija and McKenzie (17) for several technical pitfalls. The Feulgen CBA was also used to detect thyroid growth stimulating antibodies (TGI) in nonautoimmune conditions such as sporadic nontoxic goiter (14) or endemic goiter (18), but its results have been considered with much concern (16, 17). This method is based on a time-dependent procedure for quantification of cellular DNA content in guinea pig thyroid segments. Cells having more than 1.4 times the normal DNA are assumed to be in S-phase. The basic problem with the above studies (6, 14) is that, while mammalian cells take 8–12 h to enter S-phase, an incubation time of 3–5 h with TSH or IgGs was used in the Feulgen CBA (16, 17).

In their letter, Boyages and Medeiros-Neto assume that this is not a major problem since the results of the Feulgen CBA showing TGI in endemic goiter were confirmed using FRTL-5 cells in a mitotic arrest assay (18). As discussed by Zakarija and McKenzie (17), even this confirmatory paper is a matter of uncertainity. In the latter study, IgGs from patients with endemic goiter had no effect by themselves, while they increased cell growth stimulated by 50 mU/L of TSH in a medium containing serum and insulin. These experimental conditions are not comparable to those used in the Feulgen CBA. Moreover, in the culture conditions used by Wilders-Truschnig *et al.* (18), both pathways known to mediate growth in FRTL-5 cells (*i.e.* the adenylate cyclase/cAMP cascade and the insulin/IGF-1 pathway) are already activated by TSH, serum, and insulin present in the medium. In particular, the insulin/IGF-1 pathway is fully activated (17). Thus, the effect of TGI could be exerted only through a further stimulation of the cAMP pathway. This should not be the case since TGI were always described as devoid of cAMP stimulating activity in patients with euthyroid goiter (14, 18). It is therefore difficult to envisage what growth-promoting pathway is involved in the effect of TGI (17). Moreover, similar experimental conditions have been used in our (15) and another laboratory (19) to search for TGI in IgGs from patients with endemic goiter living in Italy, Peru (15), or India (19). Both studies failed to reproduce the results of Wilders-Truschnig *et al.* (18) and, indirectly, the findings obtained with the Feulgen CBA.

As far as the preparation of IgG is concerned, we used a chromatographic separation on diethylamminoethyl-sephadex A-50 followed by concentration of IgGs by ammonium sulfate precipitation (5). Immunoelectrophoresis showed that IgG preparations were 90% pure. In her editorial to our paper, Dr. Brown (20) suggested that, while the separation step was appropriate, the concentration with ammonium sulfate could be responsible for the loss of some activity in our IgG preparations. We agree that ammonium sulfate precipitation is a rather crude method to concentrate IgGs, but the possibility that we are losing some active antibody appears remote, because IgGs from patients with autoimmune atrophic thyroiditis maintained their inhibitory effect on TSH-stimulated FRTL-5 cell growth and cAMP accumulation even when diluted to 0.1 g/L. As discussed in a previous paragraph, this dose of IgG (0.1 g/L) is similar to that reported to be effective in the Feulgen CBA for ThG-blocking Ab (6). By going further in the argument of IgG preparation,

it has been suggested (21) that, since our IgGs were only 90% pure, contamination with a growth factor or with some inhibitor could reduce the ability of our system to detect TGI in endemic goiter and ThG-blocking Ab in endemic cretins. Regarding the problem of TGI in endemic goiter, it is worth noting that, similar to us, Davies *et al.* (19) obtained negative results using protein G-separated extra pure IgGs from Indian patients with endemic goiter. The possibility that our IgGs contained nonspecific inhibitors of FRTL-5 cell growth, such as those detected in the ACTH-adrenal cell bioassay (22), is unlikely. Indeed, IgGs from normal subjects as well as from endemic cretins never produced a significant inhibition of TSH action, while a strong blocking activity was found with IgGs from patients with autoimmune atrophic thyroiditis containing TSHBAb (5).

Regarding the last point of criticism no. 3, we did not have the opportunity to document by thyroid ultrasound our palpation data in endemic cretins. We believe that this is not relevant for our conclusions, since we obtained negative results in all patients whatever the size of their thyroid and serum TSH concentrations.

4. Pathological findings in endemic cretins

In the old (1936) study by De Quervain and Wagelin (23) the main pathological findings were thyroid atrophy and fibrosis. The same picture of increased fibrous stroma with few follicles and no colloid remaining was reported by McCarrison in endemic cretins (24). On the other hand, some degree of lymphocytic infiltration of the thyroid can be found at postmortem examination in 17% of the general population without clinical evidence of thyroid disease (25). In our opinion, the occurrence of focal lymphocytic infiltration of the thyroid in a few cases of hypothyroid endemic cretins cannot be taken as proof that autoimmune aggression is the primary event leading to thyroid atrophy and hypofunction in this disorder.

In conclusion, we are confident that our negative results for ThG-blocking Ab in hypothyroid nongoitrous endemic cretins cannot be attributed to inappropriate selection of patients or methodological inadequacy. Thus, our results contradict the findings of previous studies showing the existence of ThG-blocking Ab in endemic cretins having no other thyroid autoimmune stigmata. We believe that, unless new and fully reliable evidence is provided, the hypothesis of autoimmunity as a major cause of both endemic cretinism and endemic goiter is not tenable.

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VITAMIN D RECEPTOR GENE AND OSTEOPOROSIS

To the editor:

Looney *et al.* (1) have reported on the possible relationship between the vitamin D receptor genotypes and severe osteoporosis in women. We consider some of the interpretation and conclusions may be misleading.

Their study was based on the assumption that, because vitamin D receptor (VDR) genotypes contribute to the determination of bone mineral density (BMD), they should predict osteoporosis per se. However, VDR genotypes contribute to approximately one standard deviation of BMD, which could be expected to double the risk of osteoporotic fracture. Thus, VDR genotypes could not predict severe osteoporosis or osteoporotic fracture. Furthermore, case-control studies may be confounded by selection bias, *e.g.* very low *vs.* high BMD. This can be particularly troublesome with small sample size studies.

We consider that the association between osteoporosis and VDR gene allelic variation is dependent on two factors, namely, the frequency distribution of VDR genotypes and the impact of the allele differences on the distribution of BMD. Inferences about association are often based on genotypes being in Hardy-Weinberg equilibrium in the population. However in these data, the genotype distribution in the controls deviates significantly from this equilibrium law ($P = 0.023$). Moreover, the standard deviation of the allele frequency was also higher in the controls (0.074) compared with the cases (0.054) and previous data (0.012, ref. 2); reflecting overall small sample size and particularly the smaller sample size in the control group. The most likely explanation for such differences relates to sampling bias, which can invalidate studies of marker-phenotype associations.

Thus, type II sampling error may be important in this study. Even if the prevalence of the BB genotype in the control and osteoporotic groups