Effects of *Trypanosoma congolense* infection on the pituitary gland of Baoulé bulls: immunohistochemistry of LH- and FSH-secreting cells and response of plasma LH and testosterone to combined dexamethasone and GnRH treatment

H. Boly^{1*}, P. Humblot¹, Y. Tillet² and M. Thibier¹

¹UNCELA, 13 rue Jouët, BP 65, 94703 Maisons Alfort, France; and ²Laboratoire de Neuroendocrinologie sexuelle, INRA, 37380, Nouzilly, France

The effects of *Trypanosoma congolense* infection were investigated at the pituitary level on trypanosome resistant Baoulé bulls (aged 3–6 years), using immunohistochemistry of LH- and FSH-secreting cells and a combined dexamethasone and GnRH challenge. The pituitaries of two control and five naturally infected Baoulé bulls were removed after slaughter and the LH- and FSH-secreting cells were examined immunohistochemically, using specific polyclonal antibodies against β LH and β FSH. No significant impairment of the labelling and distribution of LH- and FSH-secreting cells was seen in infected bulls when compared with control animals. No parasites were found in the pituitary glands. Plasma LH and testosterone concentrations were determined in eight control and eight infected bulls by enzymeimmunoassay and radioimmunoassay techniques, respectively. Blood samples were collected at intervals of 30 min two times before and nine times after dexamethasone treatment (20 mg i.m.). GnRH (Busereline: 20 μ g, i.m.) was injected 4.5 h later and samples were collected every 15 min for 180 min. After dexamethasone treatment, LH and testosterone concentrations declined dramatically in both groups. Four hours after treatment, the mean testosterone concentration for both groups was 0.44 ng ml⁻¹. After GnRH injection, LH concentrations in the infected group increased rapidly to a mean maximum value of 30 ng ml⁻¹ by 165 min. In contrast, the increase in LH concentration in non-infected bulls was more gradual and the mean maximum value, reached at the same time, was only 20 ng ml⁻¹. Testosterone concentration increased rapidly and in a similar manner in both groups for the first 90 min (0.08 $\pm$ 0.04 ng ml⁻¹). There was almost no further increase in testosterone concentration in the infected group (different from controls; $P < 0.05$) although LH concentrations continued to rise. The testosterone concentration of the non-infected group increased steadily, up to the end of the sampling period. It is concluded from the immunohistochemical study and from the pituitary response to GnRH that the parasites do not alter pituitary function but that they do affect testicular function.

Introduction

It is well established that trypanosome-infected cattle subsequently suffer from various kinds of reproductive disorders (Anosa and Isoun, 1980; Diabakou *et al.*, 1984; Akapvie *et al.*, 1987; Grundler *et al.*, 1988). Infected males display some alteration of sexual behaviour, and a decrease of quantitative, and qualitative, sperm parameters (Akapvie *et al.*, 1987; Sekoni *et al.*, 1988, 1990; Chicoteau, 1989; Adeyemo *et al.*, 1990; Boly *et al.*, 1991). However, the mechanisms involved in the

impairment of sexual function during trypanosomiasis infection are not well understood. The effect may be at the testicular, hypophysial or hypothalamic level.

Previous histological studies, mainly on testicular tissue (Ikede, 1979; Anosa and Isoun, 1980; Grundler *et al.*, 1988) revealed degenerative and inflammatory lesions in relation to the infectious stages and clinical symptoms. The hormonal approach based on weekly (Adeyemo *et al.*, 1990) or daily (Waidi *et al.*, 1986; Adeyemo *et al.*, 1990) blood samples or even on frequent collection of blood samples (every 15 min for 8 h: Chicoteau, 1989; Boly *et al.*, 1991) indicated some reduction in plasma concentrations of LH and testosterone. This finding suggested an impairment of pituitary function, resulting in low testicular stimulation and failure of the feedback mechanism.

*Present address: IDR, University of Ouagadougou BP 7021 Ouagadougou 03, Burkina Faso, West Africa.
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Some histological studies of the pituitary gland during infection with *Trypanosoma brucei* revealed specific lesions such as fibrosis in humans (Hawking and Greenfield, 1941) and inflammation, mononuclear infiltration and necrosis in cattle, sheep, goats, horses and dogs (Losos and Ikede, 1970, 1972; Moulton and Sollod, 1976; Ikede *et al.*, 1977; Morrison *et al.*, 1981). Nevertheless, the relationship between these lesions and the alteration of the pituitary–gonadotrophin function was not clearly demonstrated, and in particular the effects of the infection on LH- and FSH-secreting cells were not examined. Retention of gonadotrophin granules in basophil cells has recently been described in small, East African goats infected experimentally with *Trypanosoma congolense* (Mutayoba *et al.*, 1988). These authors found that pituitary function was impaired owing to either a lack of stimulation by hypothalamic neurosecretion or to a failure to release gonadotrophin; however, as GnRH affects LH synthesis and release, the precise mechanism of impairment is unknown.

The specific effects of infection on LH- and FSH-secreting cells, and their ability to release gonadotrophin hormones following dexamethasone and GnRH treatment were investigated using previously described methods (Thibier, 1976, 1977; Thibier and Rolland, 1976; Chantarapateep and Thibier, 1979; Abdel Malak and Thibier, 1982). These studies showed that dexamethasone treatment causes a fall in LH synthesis and in its release by pituitary cells, thus reducing testosterone production. The ability of pituitary cells to respond to a subsequent injection of GnRH can therefore be assessed independently from previous synthesis and release of LH and testosterone.

The aim of this study was to compare: (1) the distribution and size of LH- and FSH-secreting cells by an immunohistochemistry technique, and (2) the potential release of LH and testosterone, following combined dexamethasone and GnRH treatment in control and naturally infected Baoulé bulls.

Materials and Methods

Animals

Twenty-three adult (4–6 year-old) Baoulé bulls were purchased at the animal market of Bobo Dioulasso (Burkina Faso). They came from the Sudanese zone infected by the tsetse fly (Province of Houet, Kenedougou and Poni in Burkina Faso; 11°8'N, 4°13'W) and were raised in an extensive breeding system with a permanent pasture (MAE/SEEL, 1991). Trypanosomiasis is endemic in these animals.

Animals were divided into two groups: 13 naturally infected by *Trypanosoma congolense* and 10 non-infected controls, according to tests for parasites performed just before these investigations (Woo and Kauffmann, 1971). Jugular blood samples were collected in heparinized vacutainers (Beckton Dickinson, Grenoble) and the degree of infection was evaluated: grade 1 = low infection, that is one parasite per microscope field; grade 2 = moderate infection (2–10 trypanosomes per microscope field) and grade 3 = high infection, that is, more than 10 trypanosomes per microscope field. May-Grunwald Giemsa smears were also performed to confirm these observations and check for the presence of other parasites.

Immunohistochemistry and LH- and FSH-secreting cells

The pituitary glands from two control and five infected bulls (two with grade 1 infection, two with grade 2 and one with grade 3) were removed after slaughter, immediately fixed in Bouin–Holland solution, dehydrated in alcohol and toluene, embedded in paraffin wax and cut into thick sections of 10 µm. Four sections were analysed for each animal. After rehydration, sections were processed for immunohistochemistry according to the method of Tillet and Thibault (1987) but modified as follows. Saturation by normal sheep serum diluted 1:15 in 0.1 mol PBS l⁻¹, pH 7.4 was performed for 20 min. Consecutive sections were incubated overnight at 4°C in anti-LH (specific to β subunit), anti-FSH (specific to β subunit) and a nonimmune control serum raised in rabbits and diluted 1:500 in PBS and 0.1% BSA. Sections were subsequently incubated for 2 h at room temperature (22°C) with a second serum anti-rabbit γ globulin diluted 1:100 in PBS. Immunoperoxidase staining was then carried out by incubating the sections for 2 h with rabbit peroxidase–anti-peroxidase complex diluted 1:200 in PBS. Peroxidase activity was revealed within a few minutes, in a mixture of 0.02% of 3,3' diaminebenzidine hydrochloride, 0.003% H₂O₂ (110 vol.) in 0.05 mol Tris–HCl l⁻¹, pH 7.9. Sections were rinsed three times in 0.1 mol PBS l⁻¹ between each step. The specificity and characteristics of the antisera have been described by Dubois (1971) and Dacheux and Dubois (1976), and the specificity of the reaction was controlled with the nonimmune serum. The distribution, mean size and number of LH- and FSH-secreting cells were measured twice in 20 microscope fields.

Endocrine study

For LH and testosterone assays, blood samples were taken from the jugular vein of eight control and eight infected bulls using heparinized vacutainers (Beckton–Dickinson), according to the method described by Thibier and Rolland (1976) and Abdel-Malak and Thibier (1982).

Blood samples were taken 30 min before and at the time of an i.m. injection of 20 mg dexamethasone (Dexadren: Intervet, Angers). Nine further samples were taken at intervals of 30 min. Synthetic GnRH (Busereline: Distrivet, Paris) was then administered (20 µg i.m.). Fourteen blood samples were then taken at intervals of 15 min. After collection, blood was centrifuged (1500 g, 10 min), and plasma immediately decanted and frozen (–20°C) until assayed.

Plasma LH concentrations were determined using an enzymeimmunoassay according to the procedure described by Maurel (1991). This assay kit, supplied by SANOFI Santé Animale (Libourne, France), involves two anti-LH polyclonal antibodies which bind LH. A third antibody, labelled with peroxidase, reveals LH binding during a coloured reaction using a chromogen (ABTS). Samples were successively incubated for 45 min with the first antibody coated on microplates and the second antibody. The conjugate was then added and incubation was continued for 45 min. The absorbance (405 nm) was measured 1 h after the addition of chromogen to a microplate reader (Dynatech MR 5000). Between each step, the plates were washed five times with distilled water. As reported by Maurel (1991), crossreactivity with bovine LH and

correlation with a previously described specific radioimmunoassay was excellent ($> 95\%$). For the assays in the present study, the limit of detection was lower than 0.1 ng ml^{-1} ($P < 0.01$), and the intra- and interassay coefficients of variation for a 9 ng ml^{-1} sample were 3% and 10%, respectively.

Plasma testosterone concentrations were determined by radioimmunoassay as described by Thibier (1975). Briefly 40 000 d.p.m. of tritiated testosterone (Radiochemical Centre, Amersham) were added to 0.5 or 1.0 ml of plasma. After ether extraction and Sephadex LH 20 microcolumn chromatography, the testosterone was incubated (30 min at 37°C) with a specific antiserum (number 2094S2; generously supplied by J. Adeline, Fondation de recherche en hormonologie, 94260 Fresnes, France). Free steroids were separated from bound steroids by the toluene method at a constant temperature (18°C). Intra- and interassay coefficients of variation were 10.2% and 11.6%, respectively, and the limit of detection was 0.03 ng ml^{-1} .

Statistical analysis

Data were recorded and analysed with SAS analysis system (1987). The effects of infection on LH- and FSH-secreting cells, determined by immunohistochemistry, were evaluated by ANOVA using proc GLM. Mean LH and testosterone concentrations were compared in control and infected animals at various times using Student's *t* test. In addition, mean areas under the curve, calculated during three periods ($\text{ng ml}^{-1} \times 30 \text{ min}$ before administration of dexamethasone, $\text{ng ml}^{-1} \times 270 \text{ min}$ after dexamethasone and $\text{ng ml}^{-1} \times 180 \text{ min}$ after synthetic GnRH), were compared in the two groups and the correlation between areas evaluated. Results were considered significantly different if $P < 0.05$.

Results

Immunohistochemistry

The immunoperoxidase labelling revealed positive staining of LH- and FSH-secreting cells which appeared homogeneous in the cytoplasm. The intensity of staining was more pronounced in LH-secreting cells than with FSH-secreting cells (Fig. 1a, b). All control non-immune serum samples gave negative staining. LH-secreting cells were evenly distributed in the central and peripheral part of the gland and occupied 4.1% of the anterior hypophysis. FSH-secreting cells occupied about 0.6% of the anterior hypophysis and were mainly distributed in the peripheral part of the gland with only slight staining in the central part compared with LH staining. There was no difference in the labelling or distribution of LH- and FSH-secreting cells between control and infected bulls. There was, however, a tendency for more intense staining of the LH-secreting cells of the infected bulls (Fig. 1c, e) than in control (Fig. 1a). Moreover, the mean cell size was significantly greater ($P < 0.05$) in highly infected (grade 3) bulls ($481 \pm 47 \text{ nm}^2$) compared with the controls ($380 \pm 32 \text{ nm}^2$) (Fig. 1a, e). No parasites were found in the pituitary gland.

Endocrine study

Before dexamethasone injection, the mean LH concentrations were $0.1 \pm 0.05 \text{ ng ml}^{-1}$ for control and $0.12 \pm 0.04 \text{ ng ml}^{-1}$ for infected bulls ($P > 0.05$). Concentrations of testosterone were of $5.53 \pm 3.88 \text{ ng ml}^{-1}$ and $0.61 \pm 0.38 \text{ ng ml}^{-1}$ ($P < 0.05$) in controls and infected animals, respectively.

One hour after dexamethasone treatment, LH concentrations had decreased below the limit of detection of the assay in most animals (12 of 16; 75%), and no pulsatility was observed during the 270 min of sampling (Fig. 2a). During this period no significant difference was found between LH concentrations of infected and control bulls. By contrast, testosterone concentrations decreased markedly in the non-infected bulls to a low mean concentration of $0.44 \pm 0.35 \text{ ng ml}^{-1}$ 4 h later, whereas those of the infected animals did not vary and remained low during this period ($0.33 \pm 0.16 \text{ ng ml}^{-1}$).

After GnRH injection, LH concentrations in the infected group increased rapidly to a mean maximum value of 30 ng ml^{-1} 165 min later and then declined. By contrast, the non-infected bulls presented a more gradual increase and the maximum mean value reached at the same time, was only 20 ng ml^{-1} . During the first 90 min, the rates of increase were $0.27 \pm 0.1 \text{ ng ml}^{-1} \text{ min}^{-1}$ and $0.07 \pm 0.03 \text{ ng ml}^{-1} \text{ min}^{-1}$, respectively, for infected and control animals (Fig. 2a). The mean areas under the curve during the 240 min after GnRH injection were $3370.57 \pm 1467.81 \text{ ng ml}^{-1}$ and $2212.31 \pm 805.96 \text{ ng ml}^{-1}$ ($P < 0.05$) for the infected bulls and the control bulls, respectively. The LH response after GnRH correlated with the LH concentration before dexamethasone injection, $r = 0.46$ ($P < 0.05$).

Testosterone patterns after injection of GnRH differed from those of LH (Fig. 2b). Concentrations increased rapidly and similarly for the first 90 min ($0.08 \pm 0.04 \text{ ng ml}^{-1} \text{ min}^{-1}$) in both groups. However, after this time there was almost no further increase in testosterone concentration in the infected group even though LH concentrations continued to increase. In the non-infected group, testosterone concentrations increased steadily up to the end of the sampling period. The mean areas under the curve were not significantly different ($P > 0.05$) between control and infected bulls despite the high testosterone concentration found in control animals: $2120.84 \pm 1163 \text{ ng ml}^{-1} \times 240 \text{ min}$ and $1055.45 \pm 981 \text{ ng ml}^{-1} \times 240 \text{ min}$, respectively. The magnitude of the increase of testosterone concentrations correlated with the concentration before dexamethasone treatment ($r = 0.72$; $P < 0.002$) and negatively with the degree of infection ($r = -0.64$; $P < 0.01$).

Discussion

The results of immunohistological labelling of LH- and FSH-secreting cells were similar to those reported by Dubois (1969, 1971), Dacheux and Dubois (1976) and Tillet *et al.* (1990). LH-secreting cells stained more strongly than did FSH-secreting cells. As reported by Combarnous (1991), the number of LH-secreting cells was about seven times higher (4.1% versus 0.6%) than that of FSH-secreting cells. Infected bulls showed no difference in cell labelling and distribution,

Fig. 1. Immunoperoxidase staining of pituitary cells from Baoulé bulls labelled with (a,c,e) anti-LH serum and (b,d,f) anti-FSH serum. (a,b) Cells from non-infected, control bulls. Cells from bulls with (c) moderate, (d) low and (e,f) high degrees of trypanosome infection. Cells from non-infected bulls were only lightly stained for LH and FSH. Intensity of staining increased with degree of infection and there was a slight increase in cell area (e,f) when the degree of infection was high. Bar represents 100 μ m.

suggesting that the infection has no direct pathological effect on the pituitary gland. This contention is supported by the absence of parasite and specific lesions as described during *Trypanosoma brucei* infection by other workers (Losos and Ikede, 1970, 1972; Moulton and Sollod, 1976; Ikede *et al.*, 1977; Morrison *et al.*, 1981). Although only one case of high infection (grade 3) was found from the group of naturally infected bulls, an increase in mean size of LH-secreting cells was

observed and resembled the retained gonadotrophin granules described by Mutoyoba (1988).

Before the injection of GnRH, that is, before and after dexamethasone treatment, LH patterns in both groups were similar to those reported in bulls (Thibier, 1975; Schanbacher and Echternkamp, 1978; Chantaraprateep and Thibier, 1979; Barnes *et al.*, 1981; Abdel Malak and Thibier, 1982). After the dexamethasone injection, LH concentrations decreased in both

Fig. 2. Mean ($\pm$ SEM) concentrations of (a) LH and (b) testosterone in (○) controls and (●) bulls infected with trypanosomes, before and after dexamethasone treatment, and after the GnRH challenge. Arrows indicate times of dexamethasone (DXM) and GnRH injections.

groups as anticipated. At the onset of the sampling period, testosterone concentrations were high in non-infected bulls and after dexamethasone treatment concentrations decreased dramatically. In this respect, trypanosome resistant Baoulé bulls (*Bos taurus*) in tropical areas behave in a similar manner to European cattle (Thibier and Rolland, 1976). By contrast, infected bulls had low testosterone concentrations. This finding is consistent with previous reports (Chicoteau, 1989; Adeyemo *et al.*, 1990; Boly *et al.*, 1991). However, the two blood samples taken 30 min before dexamethasone injection were not sufficient to differentiate significantly between the testosterone concentrations of control and infected bulls. There was a marked reduction in the infected individuals, which is also consistent with the findings of Adeyemo *et al.* (1990).

After GnRH injection, LH was released at a much higher rate in infected bulls than in non-infected animals. In the study reported here, non-infected animals had lower peak concentrations than did those reported by Thibier (1975); however, factors such as breed and environmental conditions may explain such differences.

After the GnRH challenge, the initial testosterone surge was identical in both groups; however, greater increases occurred in the non-infected animals than in the infected animals, where maximum values of around 8–9 ng ml⁻¹ were observed. These findings suggest that the testes of infected bulls have a lower

capacity to produce testosterone. The combination of lower values of testosterone before dexamethasone treatment and the limited surge after the GnRH challenge suggests a major impairment of the endocrine function of testicular interstitial tissue. These results are in agreement with the results of Soudan *et al.* (1992), who observed a reduction of LH receptors in mouse Leydig cells. Moreover, secretion of LH by the pituitary was not impaired in the infected group. The higher LH release and the decreased testosterone production after the GnRH challenge resembles the pseudo-castrated status described by other workers (Pelletier and Ortavant, 1972; Tannen and Convey, 1977; Schanbacher and Ford, 1977), during which the negative-feedback effect of testosterone on LH is lowered. It is also possible that an isoform of LH is released by the hypophysis of infected bulls, and this may explain the difference observed in the testicular response between the groups.

This study demonstrated, using immunohistological techniques and a combined dexamethasone and GnRH challenge, that there was no significant damage to LH- and FSH-secreting cells in trypanoresistant Baoulé bulls infected by *Trypanosoma congolense*. However, testosterone concentrations were affected in the infected animals as shown by low concentrations before dexamethasone injection and the lower release after the GnRH-induced LH stimulation when compared with non-infected animals. The low testosterone concentration and the limitation of the magnitude of this testosterone surge, despite the high LH concentrations after the GnRH challenge, suggests that an alteration of testicular interstitial tissue or a modification of LH bioactivity occurs after infection.

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