ELSEVIER

Acta Tropica 72 (1999) 137–148

ACTA TROPICA

Isolation of *Trypanosoma brucei* from the monitor lizard (*Varanus niloticus*) in an endemic focus of rhodesian sleeping sickness in Kenya

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Received 20 May 1998; received in revised form 12 August 1998; accepted 19 October 1998

Abstract

Monitor lizards were sampled along the shores of Lake Victoria to detect natural infections of potentially human-infective trypanosomes. In an area with endemic rhodesian sleeping sickness, one of 19 lizards was infected (Busia, Kenya). Six of ten lizards also showed indirect evidence of infection with *Trypanosoma brucei* (antibody ELISA). In an area with no recent history of human disease (Rusinga Island), no parasites were found and no antibodies to *T. brucei* were detected. The isolate was identified as *T. brucei* through xenodiagnosis (completion of the life cycle in the salivary glands of tsetse), and through molecular techniques (positive reactions with a PCR primer and a microsatellite DNA probe characteristic of the subgenus *Trypanozoon*). Experimental infections of monitor lizards were also attempted with a variety of parasites and tsetse species. It was possible to infect monitor lizards with *T. brucei* but not with forest or savannah genotypes of *Trypanosoma congolense*. Parasites reached low levels of parasitaemia for a short period without generating any pathology; they also remained infective to tsetse and laboratory rats. The implications of these findings are discussed in relation to the endemicity of sleeping sickness.

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0001-706X/99/$ - see front matter
PII: S0001-706X(98)00092-8

Keywords: *Varanus niloticus*; *Trypanosoma brucei*; *Trypanosoma congolense*; *Glossina fuscipes*; Reservoirs; Sleeping sickness

1. Introduction

The shores of Lake Victoria have been endemic foci for human sleeping sickness since the early 1900s (Hide et al., 1996). *Glossina fuscipes* has been the major vector in historical and ongoing epidemics in Uganda (Okoth and Kapaata, 1988). In Kenya, *G. pallidipes* has been responsible for recent epidemics in the Lambwe Valley, as well an ongoing outbreak in Busia (Angus et al., 1995). Recognised reservoirs for the disease are wild mammals such as the bushbuck (Heisch et al., 1958) and domestic animals, particularly cattle (Onyango et al., 1966; Maudlin et al., 1990). Tsetse bloodmeal analyses from the region and elsewhere indicate that *G. fuscipes* feeds primarily on reptiles such as the Nile monitor lizard, *Varanus niloticus* (Mohamed-Ahmed and Odulaja, 1997). This is in contrast to the feeding habits of *G. pallidipes*, which prefers to feed on a variety of wild and domestic mammals. As a result of human population growth, there is now close contact between man, livestock and reptiles at watering points along rivers, streams and the lakeshore. *G. fuscipes* adapts easily to these peridomestic habitats, providing an important link between diverse hosts.

Except for Woo and Soltys (1969) and Molyneux (1973), minimal work has been carried out to determine the role of African reptiles as reservoirs of sleeping sickness. It is not clear whether critical hosts of riverine tsetse harbour trypanosomes pathogenic to man or to livestock. Diagnostic studies of monitor lizards were therefore carried out at two contrasting locations along the shores of Lake Victoria: Busia, an endemic focus and Mbita Point (Rusinga Island) an area with no recent history of disease. The objective was to determine the natural occurrence of trypanosomes in lizards in areas with contrasting epidemiology. The field investigation was supplemented by attempts to infect lizards experimentally with *Trypanosoma brucei* and *T. congolense* by tsetse challenge.

2. Material and methods

2.1. Tsetse flies

Glossina morsitans centralis, *G. fuscipes*, *G. palpalis gambiensis* and *G. tachinoides* teneral males were used, usually the day after emergence. *Glossina m. centralis* was obtained from the International Centre of Insect Physiology and Ecology (ICIPE, Nairobi). *Glossina fuscipes* was obtained from the International Atomic Energy Agency (IAEA, Vienna). *Glossina tachinoides* and *G. p. gam-*

biensis were obtained from the Centre International de Recherche-Développement sur l'Elevage en Zone subhumide (CIRDES, Bobo-Dioulasso). Flies were maintained at 25 ± 1°C, 65–85% relative humidity, and 12 L: 12 D.

2.2. Trypanosomes

Lizards were infected through tsetse challenge with a variety of well-characterised parasite clones of tsetse origin: *T. brucei* (Ng3) and savannah *T. congolense* (Ng5), both originating from mature infections in *G. pallidipes* at Ngulia in Tsavo West National Park, Kenya (Mihok et al., 1992); and forest *T. congolense* (ANR3) originating from an immature infection in *G. p. gambiensis* at Abuko in The Gambia (McNamara and Snow, 1991). *T. brucei* Ng3 is killed when incubated in human serum in the BIIT test (Njagu, 1998), but its status in terms of human infectivity is unknown (Ngulia is far from any areas with human sleeping sickness). All parasites were first expanded in goats using *G. m. centralis* as the vector. Stabilates were then prepared in liquid nitrogen to provide standard material for infecting tsetse.

2.3. Monitor lizards

Monitor lizards were obtained from the shores of Lake Victoria in Kenya at two locations: Rusinga Island (0° 25′ S latitude and 34° 10′ E longitude) near Mbita Point, and Busia District at Sio Port (70 km due north of Rusinga Island on the opposite shore). Traps baited with fish were used to catch animals. The lizards were restrained manually; blood was obtained from the ventral tail vein using a 19 G $1\frac{1}{2}$ in. needle as described by Esra et al. (1975). Lizards were transferred to Nairobi, where they were kept at ambient temperature and fed on Nile perch. Only lizards captured from Rusinga Island were used in experiments, usually within a few days of capture.

2.4. Diagnostic techniques

2.4.1. Parasite detection

No single technique meets all the requirements for the detection of trypanosomes in wildlife (Dillmann and Townsend, 1979; Truc et al., 1997). A battery of techniques was therefore used: thin, thick, and wet smears; dark ground buffy coat examination (Paris et al., 1982); the kit for in vitro isolation (KIVI, Aerts et al., 1992; Truc et al., 1992); inoculation of blood into cyclophosphamide-immunosuppressed Wistar rats (0.5 ml into each of five rats, lizard blood even in minute quantities is lethal to Swiss or Balb/c mice, Njagu, 1998), and xenodiagnosis using susceptible tsetse (50 male *G. m. centralis* fed on a 1:4 dilution of lizard blood in fresh goat blood; goat blood facilitates trypanosome establishment in this tsetse species, Mihok et al., 1995). With a few exceptions, all techniques were used for the diagnosis of each wild lizard. The identity of the single wild isolate was confirmed with the polymerase chain reaction (PCR) and by hybridisation with DNA probes

(Masiga et al., 1992; Majiwa et al., 1994). Full details of methods can be found in Njagu (1998).

2.4.2. Antibody detection

During blood collection for parasitology, we also collected small quantities of serum from some of the wild lizards for serology. These were tested in an antibody ELISA using protein-A peroxidase as a conjugate. As this was the first ever test with lizard serum, there were no guidelines for cut-off values. For coating the plate, purified variable surface glycoprotein of *T. b. gambiense* variable antigen type LiTat 1.3 (Lejon et al., 1998) was diluted in PBS to a concentration of 2 μg/ml. The antigen solution was dispensed at 150 μl/well in a Nunc-immunoplate Maxisorp and incubated overnight at 4°C. Half of the wells were left empty as an antigen negative control. Further manipulations were according to Büscher et al. (1995) except:

Serum dilution: A 1/200 dilution of serum in PBS-Blotto was added to both antigen-containing and control wells, at 150 μl per well. The plate was incubated for 30 min at room temperature.

Conjugate incubation: Protein A-peroxidase conjugate (Sigma 1 mg Lot 106H8280) diluted 1/5000 in PBS-Tween was added at 150 μl per well and incubated for 1 h at room temperature.

Colour development: An ABTS tablet (Boehringer 1112422, 50 mg/tablet) was dissolved in 100 ml ABTS buffer and added at 150 μl/well. Incubation was at room temperature for 1 h.

2.5. Infection of lizards

Morsitans (*G. m. centralis*) and *palpalis* (*G. fuscipes*, *G. p. gambiensis*, *G. tachinoides*) tsetse with metacyclic infections (detected in advance by examining saliva from flies probing on warm slides) were allowed to feed freely on recently-captured lizards diagnosed as being free of trypanosomes. Infection was initiated by releasing flies into a large cage containing a freely-moving lizard. The flies were later collected and dissected to confirm the numbers feeding and the presence of metacyclic trypanosomes. Due to variation in maturation rates in tsetse, and the logistics of obtaining both lizards and tsetse flies at the same time, lizards were challenged by different numbers of infective bites in each experiment.

Infection status was monitored by microscopy (buffy coat technique and smears) every few days for 30 days using blood obtained through toe-clipping. On the day lizards became patent, larger quantities were collected for rat inoculation and xenodiagnosis. This was done to confirm that the parasite had retained its ability to infect mammals and tsetse. Rat inoculation and xenodiagnosis were also performed on day 30 for the lizards that never became patent.

3. Results

3.1. Diagnosis of wild lizards

Twenty-seven lizards were caught from Rusinga Island and 19 were caught from Busia over a period of $1\frac{1}{2}$ years. Sampling was continuous at Rusinga Island, but only one visit was made to Busia in November 1996. No trypanosomes were detected in any lizard from Rusinga Island. One lizard from Busia (5.3%) was diagnosed parasitologically positive. The parasites were detected by growth in rats and through xenodiagnosis, but not through the other techniques. Out of five rats inoculated with fresh blood from the one positive lizard, three had apparent *T. brucei* infections 11, 15 and 17 days later. Xenodiagnosis with *G. m. centralis* revealed procyclic trypanosomes in 6.8% ($N = 44$) of the flies dissected 6 days later.

Parasites were isolated a second time from the same lizard in three rats using blood cryopreserved directly in the field. DNA from this parasite population was purified from rat blood by DEAE cellulose column chromatography, phenol extraction and ethanol precipitation. The material produced a PCR amplification product of 800 bp (Fig. 1A) using the *T. brucei* primer ILO342 (Majiwa et al., 1994). The product hybridised with the DNA probe *pgDRI* (a portion of *ingi*); this probe is specific for the *Trypanozoon* subgenus (which includes *T. brucei*, Fig. 1B). When the same stock from rats was fed to *G. m. centralis* (mixed 1:4 with goat

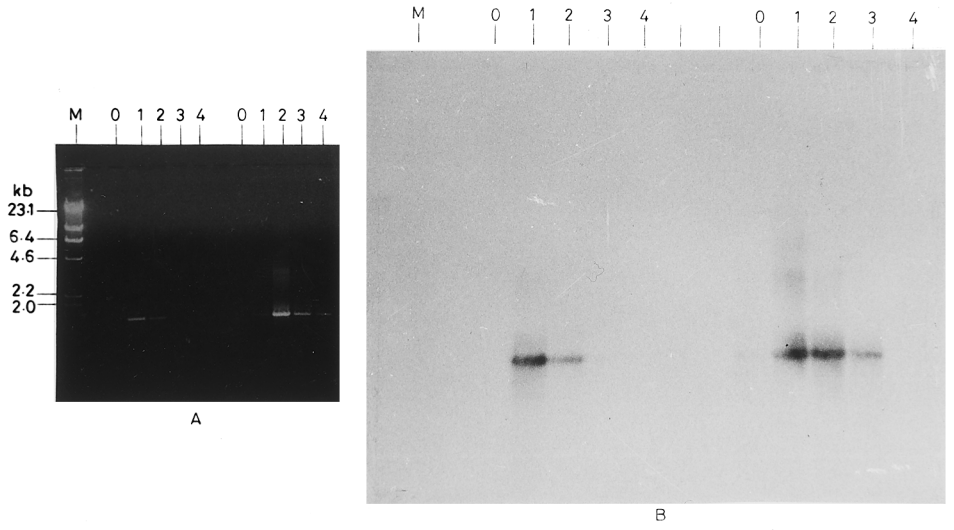

Fig. 1. (A) Agarose gel stained with ethidium bromide showing an 800 bp PCR amplification product (from primer ILO342). Target DNA was produced from DEAE purified trypanosomes from rats infected with cryopreserved monitor lizard blood (Lane M, molecular weight markers, Lane 0, negative control, Lanes 1–3, serial 10-fold dilutions of target DNA, in duplicate). (B) Autoradiograph showing hybridisation of the *Trypanozoon* specific DNA probe pgDRI with the 800 bp PCR amplification product.

blood), infective metacyclics were found in the salivary glands in 10.5% of 38 flies dissected on day 35 post infection. Salivary gland infections were also produced with this stock in all three *palpalis* tsetse (7.7% *G. fuscipes* ($N = 26$), 12.5% *G. tachinoides* ($N = 32$), 10.7% *G. p. gambiensis* ($N = 28$)).

3.2. Antibody ELISA

Ten serum samples from Busia including the parasitologically positive lizard and 17 samples from Rusinga Island were tested with ELISA. The OD range in the antigen negative controls was 0.030–0.215. The 17 samples from Rusinga Island had similar OD values between 0.041 and 0.184; hence they were all considered negative. Six out of ten sera from Busia, including the sample from the parasitologically positive lizard, had OD values from 0.332 to >3 and were considered positive; the other four had OD values less than 0.086 and were considered negative.

3.3. Experimental infection of lizards

T. brucei Ng3 was transmitted successfully to three lizards using from 6 to 17 infective bites delivered by *G. m. centralis*, *G. fuscipes* or *G. tachinoides* (Table 1). One attempt to infect a lizard with ten bites from *G. palpalis gambiensis* probably failed as no parasites were detected by any technique for one month. In the three successful transmissions, parasitaemia was never high, with only one to five parasites counted per buffy coat ($N = 19$, mean 3.2). This translates to about ten to 60 parasites/ml based on the volume sampled from the microhematocrit tube. However, due to the sensitivity and reliability of counting, actual parasitaemia was probably much higher (10^3–10^4 per ml, for counts of up to ten parasites per preparation, Paris et al., 1982).

The lizards became patent between days 8 and 14 and infections remained patent for about 2 weeks. *T. brucei* in all three lizards was infective to tsetse and rats when parasites first appeared in the circulation (Table 1). The lizards either self-cured, or parasites remained below limits of detection, as they remained negative on microscopic examination until observations were discontinued. Unfortunately, no terminal xenodiagnosis was performed in the three positive lizards, and hence their final infection status was uncertain.

One of the infected lizards died unexpectedly on day 28, probably due to problems of feeding (infected lizards were reluctant to eat and were lethargic). Otherwise, there were only minor indications of pathology. Two lizards showed modest declines in packed cell volume (PCV), typical of trypanosomosis (Fig. 2). These low PCVs were outside the range of 90 values recorded from other lizards that never showed signs of infection. More significantly, body temperatures in all three lizards increased progressively after infection until about day 20 to levels mostly higher than those recorded in lizards that never became infected (Fig. 3). Body temperatures also fell when parasites disappeared from circulation (Fig. 3).

Table 1
Diagnosis of infection in monitor lizards (*Varanus niloticus*) infected experimentally with *Trypanosoma brucei* by *Glossina* challenge[a]

Species initiating infection and number of infective bites	Duration of parasitaemia revealed by buffy coat technique	Inoculation of immunosuppressed rats	Xenodiagnosis with *G. m. centralis*	Endpoint on day 30[b]
G. morsitans centralis 17	Days 8–20	Day 8, three of three rats positive with pp of 7 days	Day 8, 9% of 42 flies positive	Died on day 28 while negative
G. fuscipes 6	Days 12–21	Day 12, two of three rats positive with pp of 9 days	Day 12, 19% of 32 flies positive	Healthy, negative
G. tachinoides 11	Days 14–26	Day 14, two of three rats positive with pp of 10 days	Day 14, 21% of 38 flies positive	Healthy, negative
G. palpalis gambiensis 10	Negative	Day 30, negative	Day 30, negative	Healthy, negative

[a] pp, prepatent period.
[b] Negative endpoints at 1 month in the three positive lizards are based only on the absence of parasites during the last few microscopic examinations of buffy coat preparations.

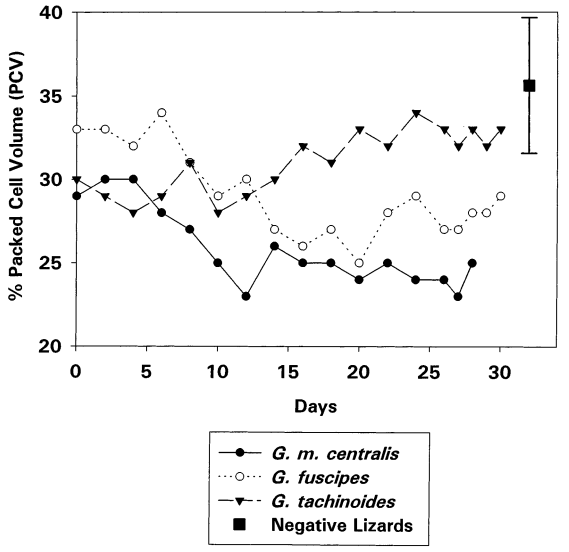

Fig. 2. Packed cell volumes (PCV) of individual monitor lizards (*Varanus niloticus*) infected with *Trypanosoma brucei* by fly bite with three tsetse species on day 0. The mean ± 2 S.D. ($N = 90$) of lizards not showing signs of infection in unsuccessful attempts is plotted for comparison on the right.

Three attempts to infect lizards with savannah *T. congolense* using *G. m. centralis* (12 bites), *G. p. gambiensis* (ten bites) and *G. f. fuscipes* (four bites) failed to produce a patent infection, as did two attempts with forest *T. congolense* using *G. tachinoides* (eight bites) and *G. p. gambiensis* (seven bites). In all of these failed attempts, we observed no indications of cryptic infection in terms of changes in PCV or rectal temperature. Similarly, on day 30 post infection, we failed to detect parasites using either rat inoculation or xenodiagnosis.

4. Discussion

4.1. Parasite isolation

Although many techniques were used for diagnosis, the single isolate from Busia was revealed only through rat inoculation and xenodiagnosis. KIVI, whose efficiency is remarkable at detecting trypanosomes in West Africa (Aerts et al., 1992; Truc et al., 1992, 1997), detected no infections. We used the anticomplementary anticoagulant (sodium polyanetholesulphonate) and also ran positive controls (inoculation of culture with parasitaemic blood from a rodent); hence the test should have detected parasites if they were present. KIVI also performed poorly in attempts to isolate *T. brucei gambiense* from humans in Uganda (McNamara et al., 1995). Altogether, our results confirmed the difficulty of detecting trypanosomes in wild animals, and the need for caution in interpreting negative results. Identification

of the isolate as *T. brucei* was confirmed with DNA techniques, and through development in the salivary glands of four species of tsetse. This is the first natural isolation of *T. brucei* from any reptile. Its affinity to the subspecies of *T. brucei* (*brucei*, *rhodesiense*, *gambiense*) is unknown, but will be the subject of further investigations.

Tsetse in the *palpalis* group feed on reptiles other than the monitor lizard; hence surveys for trypanosomes can be complicated by the presence of stercorarian trypanosomes such as the crocodilian parasite, *T. grayi* (Gouteux and Gibson, 1996) and the varanid parasite *T. varani* (Minter-Goedbloed et al., 1993). These parasites are distinct biochemically (Dirie et al., 1991; Haag et al., 1998), but can be confused with immature infections of pathogenic trypanosomes in tsetse. In this study, despite the fact that culture and xenodiagnosis techniques were used, no unusual organisms were found. To date, no-one has been particularly successful in isolating reptilian trypanosomes (Minter-Goedbloed et al., 1993; Solano et al., 1996).

Lizards were susceptible to tsetse challenge with *T. brucei* but not with *T. congolense*. These trends agree with the refractoriness of the riverine group for *T. congolense* (Reifenberg et al., 1996; Njagu, 1998). Although successful in infecting lizards with *T. brucei*, infections were detectable for a few weeks only, and only at low parasitaemia. Other attempts to infect reptiles with *T. brucei* have also been successful but have used artificial conditions (syringe challenge) and odd parasite stocks (laboratory-adapted strains) as well as inappropriate hosts (ones not found

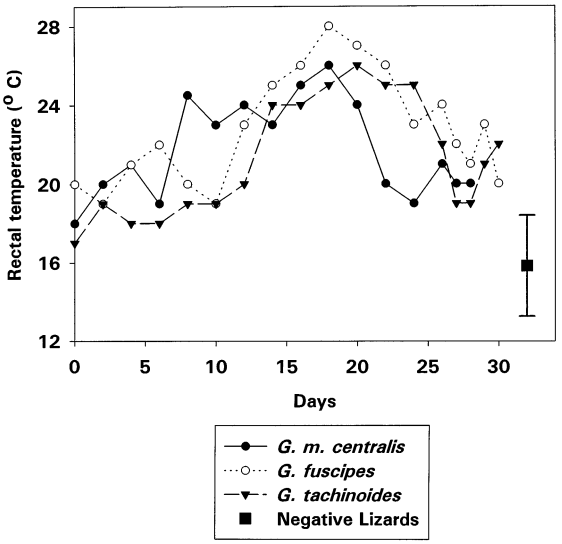

Fig. 3. Rectal temperatures of individual monitor lizards (*Varanus niloticus*) infected with *Trypanosoma brucei* by fly bite with three tsetse species on day 0 (ambient temperatures at the time of measurement were about 16–20°C). The mean ± 2 SD ($N = 90$) of lizards not showing signs of infection in unsuccessful attempts is plotted for comparison on the right.

in Africa). For example, Woo and Soltys (1969) were able to demonstrate that American caymans can support infections of *T. brucei*. Molyneux (1973) also reported on unpublished attempts to infect lizards, snakes and tortoises. He was able to establish and passage long-term infections in certain species, especially *Agama* lizards. In more appropriate hosts, he reported that *V. niloticus* and *V. exanthematicus* could support transient infections of *T. b. brucei* and *T. b. gambiense* (syringe challenge of rodent-adapted stocks). These experiments all differ from natural challenge, which involves expansion of insect metacyclic forms, followed by transformation into vertebrate bloodstream forms, and finally sustained multiplication in the host. These conditions were replicated here using appropriate parasites, vectors and hosts, but unfortunately, without control over potentially important environmental conditions.

Radiotelemetry has shown that Australian semi-aquatic varanids maintain high body temperatures at low ambient temperatures through behavioural means (Christian and Weavers, 1996). In captivity in Khartoum, *V. niloticus* maintained a mean basking/activity temperature of 31.9°C in the only similar study conducted in Africa (Hirth and Latif, 1979). In our study, lizards were infected and confined at 16–26°C with few opportunities to boost body temperature. These conditions were probably suboptimal for both the parasite and the host. In natural lizard habitat at Lake Victoria, temperatures are constant and high (28–33°C) and approach mammalian body temperature (37°C). Hence, until we do further work, it is unclear whether infections can be initiated and sustained under more benign husbandry conditions with just a single infective fly bite (the natural mode of transmission).

4.2. Epidemiological implications

The resurgence of sleeping sickness after many years of absence and the endemic stability of *T. brucei* strains infective to man remain an enigma (Stevens and Tibayrenc, 1996). Either the ultimate reservoir of this disease is not man, or human cases of a chronic nature are not detected and are not cured. The long-term stability of the disease is particularly well-documented for the epidemics caused by *T. brucei rhodesiense* around Lake Victoria (Wellde et al., 1989; Hide et al., 1996), despite some debate concerning historical trends (Gibson, 1996). From our preliminary results, we propose the hypothesis that reptiles may act as a cryptic pool of *T. brucei* in endemic foci, and hence provide a long-term reservoir of human-infective strains. This hypothesis has strong support in the isolation of *T. brucei* from a lizard in an endemic focus at Busia, and in the presence of seropositive lizards from the same area. In the event of an occasional feed on man or livestock, riverine tsetse such as *G. fuscipes* clearly have the potential to spark a cycle of disease in humans. Validation of this hypothesis will require careful characterisation of the potential for human infectivity of the strains of *T. brucei* found in lizards from areas of differing epidemiology, climate and fly-host contact.

Acknowledgements

We thank N. Massamba, E. Munyoki, S. Maramba and D. Mungai of ICIPE for technical assistance; I. Kaboré of CIRDES and E. Opiyo of IAEA for providing tsetse pupae, and J. McNamara of the University of Bristol for providing a stabilate of the West African parasite ANR3. This work was funded by a research grant from the European Union and represents part of the PhD thesis of Z. Njagu. The manuscript has benefited from the editorial comments of P. Büscher, for which we are grateful.

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