EXPERIMENTAL PARASITOLOGY 36, 27–33 (1974)

Trypanosoma congolense

III. Serological Responses of Experimentally Infected Cattle[1]

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(Submitted for publication April 30, 1973)

LÖTZSCH, R., AND DEINDL, G. 1974. *Trypanosoma congolense:* Experimental infection in cattle. III. Indirect fluorescent antibody and complement fixation tests to measure serological responses. *Experimental Parasitology* 36, 27–33. A complement fixation (CF) test for the diagnosis of *Trypanosome congolense* infection in cattle was developed and compared with the indirect fluorescent antibody (IFA) test.

Serological investigations were made with cattle immunized with irradiated *T. congolense* and challenged with untreated *T. congolense.* Trypanosomal antibodies were demonstrated by the CF test. The results indicated good specificity and sensitivity between the CF method and the IFA test. The CF test also afforded easier reading of results than the IFA test; however, antigen preparation was more difficult in the former.

The serological responses detected by the IFA and CF test appeared to be influenced by early variations of parasitemia, although no correlation appeared to exist between titer and parasitemia at later stages of the infection. Antibodies were detected in animals which received injections of irradiated *T. congolense* prior to challenge with viable organisms.

INDEX DESCRIPTORS: Bovine trypanosomiasis; *Trypanosoma congolense;* Fluorescent antibody; Complement fixation; Cattle; Immunity; Diagnosis.

INTRODUCTION

A fluorescence test for the detection of trypanosomal antibodies was first employed by Fife and Muschel (1959) when investigating American Trypanosomiasis. Sadun *et al.* (1963) and Wilson *et al.* (1967) showed the validity of the IFA test for the detection of trypanosomal antibodies in man in American and Africa and of Nagana antibodies in cattle, respectively. Cunningham *et al.* (1967) demonstrated the usefulness of this test in a large scale investigation of Nagana, employing a filter paper method. The value of the IFA test in the diagnosis of cattle trypanosomiasis was also demonstrated by Wilson (1969) working in Uganda and by Wiesenhütter (1969), working with experimentally infected cattle in Tanzania.

The complement fixation (CF) test has proved valuable in the diagnosis of Dourine (Mitscherlich and Wagener 1970) and of

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Chagas' diseases (Levine 1961). Schoenaers *et al.* (1953) also emphasized the usefulness of this test for the investigation of sleeping sickness in man. The latter used a *T. equiperdum* antigen and obtained 95% test accuracy. Evens *et al.* (1953) pointed out that the CF test was more reliable for the detection of trypanosome reinfection than any other test, because all others became negative much later after the treatment of sleeping sickness. Rodriguez (1964) using a *T. cruzi* antigen found CF titers of 1:5 in sera of 53 of 1770 cattle, indicating the presence of *T. guyanense* antibodies.

Other investigations were less successful with the CF test. Hornby (1932) pointed out the difficulties of the CF test when diagnosing *T. brucei* infections in the tropics. Gill (1965) described the CF test to be the least sensitive of 5 different serological tests used for the investigation of *T. evansi* infections in rats. Furthermore, Killick-Kendrick (1968) attributed to the CF test only a moderate value in the diagnosis of cattle trypanosomiasis.

While the IFA test apparently has proved of value for the detection of trypanosomiasis in cattle, reports in the literature concerning the CF test have not been as promising. It was the purpose of this investigation to conduct the CF test under controlled conditions using a purified antigen, and to compare the results with those obtained with the IFA test.

Materials and Methods

The selection of 3 groups of experimental animals and their care and infection were described previously (see Wellde *et al.* 1973). Groups I and II consisted of 8 animals (140, 151, 153, 6193, 6120, 138, 144, 162) inoculated with *T. congolense* parasitized blood and Group III consisted of 3 cattle (145, 148, 149) which had been injected with irradiated *T. congolense* before challenge. One animal (151) was treated with 7.0 mg/kg Berenil) 28 weeks postinoculation (p.i.). Blood smears were prepared and examined as previously described (Wellde *et al.* 1973).

Blood was taken from the animals weekly up to 20 weeks p.i., and thereafter, every 2 weeks. Sera were collected in graduated disPO-capillaries (0.025 ml ± ½%, Div. Amer. Hospital Supply Corp., USA), sealed with plastic powder, and stored at −20 C. Before use they were inactivated at 56 C for 30 min.

The IFA technique employed was a slight modification of that described for the detection of theilerial antibodies (Schindler and Mehlitz 1968). Lines were drawn between the areas of reaction on glass slides with a grease pencil to prevent overflowing of sera and conjugate, which were applied by a Marburg pipette (0.025 ml; F. Eppendorf, BRD).

Thin antigen smears were prepared from heparinized blood of mice (10 IU/ml) infected with *T. congolense* EATRO 1000 and were stored as described by Ross and Löhr (1968).

The fluorescein labelled rabbit antibovine–gamma globulin conjugate (Sylvana, USA) was diluted to 1:10 before use with Bacto-FA buffer (pH 7.2; Difco).

Observations were made with a Zeiss GFL fluorescence microscope fitted with an Osram HBO 200 bulb, dark field condensor, exciter filter, BG12, barrier filter 50, and 12.5 × 40 (oil) optics. The degree of fluoresence was evaluated according to method of Bailey *et al.* (1967).

The complement fixation test was described by Kolmer *et al.* (1949) but modified by the use of a microdiluter apparatus (Cooke Engineering, Alexandria, Virginia, USA). The test was performed with 2 units of preserved complement (Richardson 1941), a 2.5% suspension of sheep erythrocytes in neutral isotonic salt solution and with commercially obtained rabbit antisheep hemolysin (Behringwerke 1:6000; BRD) standardized to 4 units. After leaving the plates containing serum, complement, and antigen at 4 C overnight, the

hemolytic system was added and the plates were incubated at 37 C for 30 to 50 min, until both positive and negative controls reacted accordingly.

CF antigen was prepared from parasites isolated by the method of Lanham (1968). *T. congolense* EATRO 1000 were eluted by means of DEAE, anion exchange cellulose (DE-52 Whatman) from mouse blood, which was heparinized (10 IU/ml) and diluted 1:6 with phosphate–saline–glucose buffer (PSG) pH 8.2. The eluate was centrifuged for 10 min at 1500g and washed 3 times in PSG. The isolated trypanosomes were frozen rapidly in a dry ice–methanol bath and stored at −20 C. Twenty eluates obtained by this procedure were pooled and prepared by freezing and thawing 3 times. This stock antigen was then tested in a block titration test using reference sera.

The quality of the antigen remained unaltered up to six-fold dilution in physiologic saline. Finally, the antigen (5.0 mg protein per ml) was divided in small aliquots and stored at −20 C until use.

A preliminary test was carried out on 46 negative control sera in order to determine the baseline titer for both serological tests (Table I). The control sera had been obtained from cattle experimentally infected with *Anaplasma marginale* and *Babesia bigemina*, and from the 11 animals used in the present experiments prior to their infection.

The geometric mean of the above titers were 1:40 and < 1:10 in the IFA and CF test, respectively. Therefore, the dilutions of 1:80 and 1:10 were used as baseline titers for the 2 tests.

Results

The survival periods of the animals varied from 2–31 weeks p.i. Five animals died before the 14th week postinoculation, generally with high parasitemias. The remaining animals survived for longer periods and generally had low parasitemias at the time of death.

Figure 1 shows the IFA and CF antibody responses of the 3 animals inoculated with irradiated *T. congolense* and challenged with parasitized *T. congolense* blood. In both tests, these animals showed a rise of antibody titers during the immunization period. The initial peak of IFA and CF antibody titers of 160 and 80 were reached just prior to challenge, respectively. Both tests indicated a twofold rise of titer 2–3 weeks after challenge, and then titers leveled off at 160 and 80 in the IFA and CF test, respectively.

Since the individual reactions of the 8 animals (Groups I and II) did not differ greatly within either of the 2 serological tests, they were represented for each test by a mean response curve (Fig. 2). Individual parasitemias followed a general pattern and were also shown in one curve representing the average of the values obtained for the numbers of *T. congolense* counted in thin blood smears of those 8 animals 6 days per week.

IFA and CF mean antibody responses were almost identical, except that the for-

TABLE I

IFA and CF Titers of 46 Negative Control Cattle Sera

No. of samples	Serum dilutions							
	IFA				CF			
	<1:20	1:20	1:40	1:80	<1:10	1:10	1:20	1:40
46	2	9	26	9	43	1	—	2

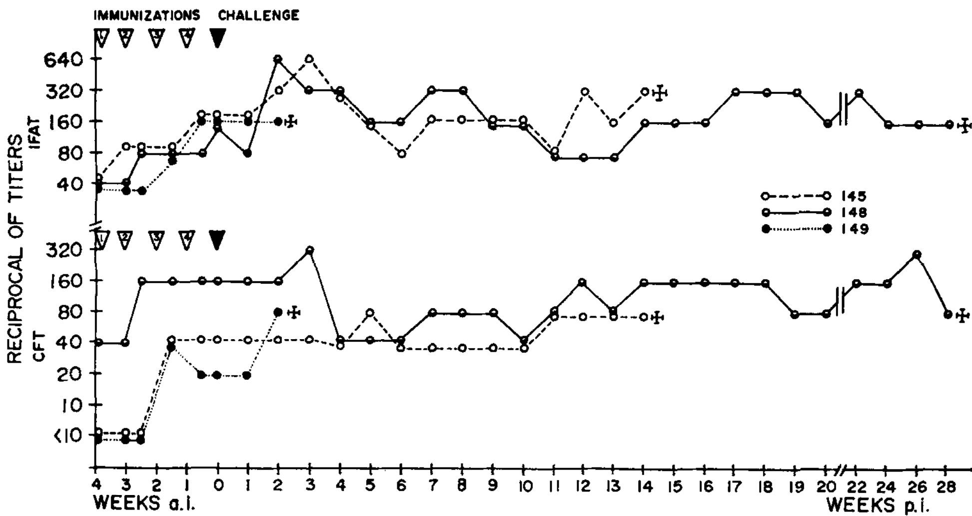

FIG. 1. The development of complement fixing and indirect fluorescent antibodies in 3 bovines (Group III) injected with irradiated trypanosomes, and subsequently challenged with viable organisms. a.i. (before inoculation), p.i. (postinoculation). Cross (✠) = animal died.

mer were on the average 1.5 dilutions lower than the latter, when reading from baselines. They reached their maximum titers (240 and 80) 3 and 2 weeks p.i., 1 week after the first rise of parasitemia. Subsequently, titers in both tests dropped one dilution and rose again to 320 and 160 in the IFA and CF test, respectively, between 7 and 12 p.i. and remained at that level.

The parasitemia was marked by a fairly consistent rise between the 2nd and 7th week p.i., a period of fluctuation between the 8th and 19th week p.i., with a maximum of 243 parasites per 10,000 erythrocytes in the 14th week p.i., and a suppressed period from the 20th week p.i. to the end.

The IFA and CF antibody titers of animal 151 started to drop 21 and 7 days, respectively, after its treatment with Berenil. Antibodies were no longer detectable in the CF test 8 weeks after treatment, whereas the IFA titer was still 160, and did not become negative until 12 weeks after treatment.

DISCUSSION

This investigation has reconfirmed the inconsistency of the appearance of *T. congolense* in the peripheral blood vessels (Hornby and Bailey 1931). The diagnosis of *T. congolense* infection by means of microscopy is sometimes not reliable because of this inconsisitency, especially in the latter stages of the infection (from 14 weeks p.i. onwards). Specific antibodies to *T. congolense*, however, can be detected continuously in the IFA and CF tests from 14 days p.i.

Initially, the serological response in both tests was apparently related to the degree of parasitemia. The first antibody titers of diagnostic importance of 320 and 80 in the IFA and CF test, respectively, appear 3 and 2 weeks p.i., after the first rise of parasitemia during the second week of infection. Also, the generally consistent rise of the parasitemia up to week 7 p.i., was associated with the next rise of titer. But after IFA and CF titers had reached levels of 320 and 160, respectively, they were not influenced by further changes of parasitemia, which agrees with the findings of Wiesenhütter (1969) and Schindler and Sachs (1970). This would indicate that both serological tests detect common antibodies, and not antibodies to antigenic variations of *T. congolense* as the neutrali-

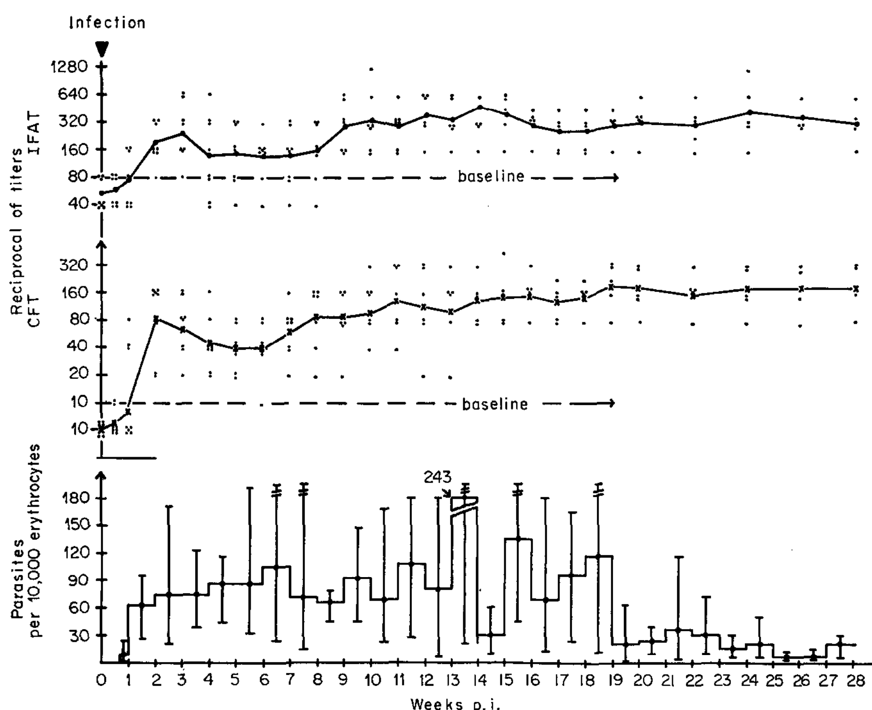

FIG. 2. The development of complement fixing and indirect fluorescent antibodies in 8 bovines (Groups I and II), infected with *Trypanosoma congolense*. Cross (✠) = animal died.

zation test appears to do (Wilson, 1968). The production of these common antibodies after 11 weeks p.i. apparently was dependent on the presence, but not on the number of trypanosomes in the host. After elimination of the parasites from the hosts, serological antibody production decreased rapidly as shown by the rapid fall of the antibody titer in animal 151 after treatment with Berenil. Since the CF titer of this animal dropped below a detectable level a very short time after its treatment, the CF test may be considered fairly reliable not only for the detection of reinfections of human sleeping sickness (Evens *et al.* 1953), but also of Nagana.

Although on the one hand the injections of irradiated *T. congolense* to 3 animals effected a rise of antibody titer which was detected significantly in the CF test 1 week earlier than in the IFA test, the survival periods of these animals did not differ greatly from those of the control animals. These results may correspond to those obtained with the IFA test by Wiesenhütter (1970), who found in immunization experiments with cattle that the serological antibodies "gave no indication of the degree of immunity."

On the other hand, in the animals surviving for longer periods, the antibody titers were usually high near death, when parasitemias were suppressed to greatest degrees. Also, the prepatent time of parasitemias was extended in the immunized animals (see Part I). Although both these facts do not refute the above conclusion of Wiesenhütter (1970), it appeared that the degree of resistance in the animals against the parasites increased as the antibody levels increased.

It is interesting that the level of gamma globulin follows a pattern similar to that of the serological antibody titers (Wellde *et al.* 1973).

The sensitivity, specificity, and ease of

reading of the CF test indicates that contrary to the opinion of Killick-Kendrick (1968), the CF test can be of great value in the diagnosis of Nagana. In our opinion, these properties make this test superior to the IFA test, whose specificity was somewhat lower.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Dr. J. G. Tremlett, Chief Veterinary Research Officer, Kabete, for his liberal aid during this study. They are also grateful to the East African Trypanosomiasis Research Organization, Tororo, Uganda, for providing the strains of trypanosomes; to Dr. K.-F. Löhr, Veterinary Research Officer, Kabete, for reading the manuscript, and to Mr. I. E. Muriithi, Director of Veterinary Services, Kenya, for permission to publish this paper.

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