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COMPLEMENT-FIXING ANTIBODIES IN CATTLE EXPERIMENTALLY INFECTED WITH *THEILERIA ANNULATA* OR VACCINLTED WITH TISSUE CULTURES(*)

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SUMMARY

The complement fixation test has been applied for the detection of complement fixing antibodies in the sera of calves vaccinated against *Theileria annulata* or experimentally inoculated with virulent strains. The antigen used in the complement fixation test was freeze-dried tissue-culture grown schizonts. The incidence and persistence of complement fixing antibodies in the sera of calves after vaccination or after inoculation with wild strains has been demonstrated. The most suitable day for collecting sera from vaccinated animals to conduct a complement fixation test has been suggested.

INTRODUCTION

Methods for the detection of serological responses of animals and man, infected with protozoan parasites, have been studied by many investigators. Of the methods tested, the complement fixation (CF) test has been found to be a very specific one. The test has provided a reliable diagnostic method for dourine (Mohler, Eichhorn & Buck, 1913). Johnson & Kesler (1937) also reported the value of this test in revealing *Trypanosoma cruzi* infection in man. Kingsbury (1927) demonstrated complement fixing antibodies in human malarial infection, and promising results were reported by Eaton & Coggeshall (1939), Stratman-Thomas & Dulaney (1940) with the test in the diagnosis of malaria. Mahoney (1962) and Mahoney (1964) used the complement fixation test for the detection of antibodies in cattle infected with *Babesia bigemina* and *B. argentina.* Application of the complement fixation test to East Coast fever, which is caused by

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Theileria parua, was reported by Schindler, Wissenhütter & Mehlitz (1968). Tutushin (1967) and Konyukhov & Poluboyarova (1967) have reported the existence of complement fixing antibodies in *T. annulata* infection, using the erythrocytic forms of the parasite as the antigen.

The aim of the present experiments was to demonstrate the use of tissue-culture grown schizonts as antigen in complement fixation tests on the sera of animals vaccinated with tissue-culture (TC) vaccine and those recovered from experimental theileriasis.

MATERIALS AND METHODS

Animals. Calves used in these experiments were of 4–18 months of age. They were either of Holstein (black and white) race or from a local breed (Sarabi). The animals were shown, by microscopic examination, to be free from *T. annulata* prior to the experiments.

Strains. Four strains of *T. annulata* were used throughout the experiments:

(1) *Strain No.* 3 (*S*3). A very virulent strain which causes nearly 90 per cent mortality in pure-bred animals susceptible to theileriasis.

(2) *Strain No.* 11 (*S*11). A very mild strain which does not cause mortality in susceptible animals.

(3) *Strain No.* 15 (*S*15). A strain with medium virulence which is used for vaccine production in this laboratory.

(4) *Strain No.* 17 (*S*17). A recently isolated strain whose virulence has not been determined yet.

Antigen. Lymphoid cells containing schizonts and adapted to suspension culture according to Hooshmand-Rad and Fesharki (1968) were cultivated in Roux bottles and multiplication was allowed to continue for five days. On the fifth day the cell suspensions were pooled and centrifuged (3000 rev./min.) and the supernatant was discarded. The sediment was re-suspended in an appropriate amount of freshly prepared phosphate buffered saline (PBS) (PH 7.4) and sedimented by centrifugation as before. This was repeated twice more. The sediment from the last centrifugation was resuspended in PBS and the number of cells adjusted to 10 million/ml. This suspension was distributed in Edwards ampoules and freeze-dried. The freeze-dried antigen could be kept for a long time at 4°c. Control antigen was prepared from normal bovine lymphocytes which were treated in a similar way to the antigen.

Titration of the antigen. Two-fold dilutions of the reconstituted freeze-dried antigen were prepared in veronal buffer and were tested against a positive serum. The highest dilution giving the most fixation of complement was accepted as one unit of the antigen. Usually 1/4 dilution of the antigen was found to be the optimal dilution for the test.

Sera. Whole blood from calves was collected by jugular puncture and the serum was recovered. Sera were stored at –20°c. 1/8 dilutions of the sera were prepared in veronal buffer and inactivated at 58°c for 30 min immediately before use.

Complement titration. A series of dilutions (1/10, 1/15 ...1/40) was prepared from the stock solution (complement preserved with Witte solution at 4°c) and titration was carried out in duplicate in the test tubes. The amount of complement in the first tube showing 100 per cent lysis was defined as one unit of complement. The final titration of complement was carried out in the presence of the appropriate amount of antigen prior to every set of tests.

Complement fixation test. The test tube technique was employed but one exact unit of complement was used throughout the experiments. Three hours at 4°c followed by 30 minutes at room temperature were allowed for fixation of complement.

The test proper. Tow-fold dilutions of the inactivated sera, starting from 1/8, were prepared and 0.2 ml was put into each test tube; 0.2 ml of antigen and then 0.2 ml of the diluted complement (one unit) were added. After the tubes had been kept for 3 hours in a refrigerator and 30 min at room temperature, 0.4 ml of 2 per cent sensitized sheep red blood cells was added and tubes were transferred into a water bath at 37°c for 30 min, being shaken after 15 min. The titre of a serum was defined as the highest dilution which did not show more than 50 per cent haemolysis. Antigen, complement, red cells fragility controls and at least one known negative serum and one positive serum of known titre were included in each test.

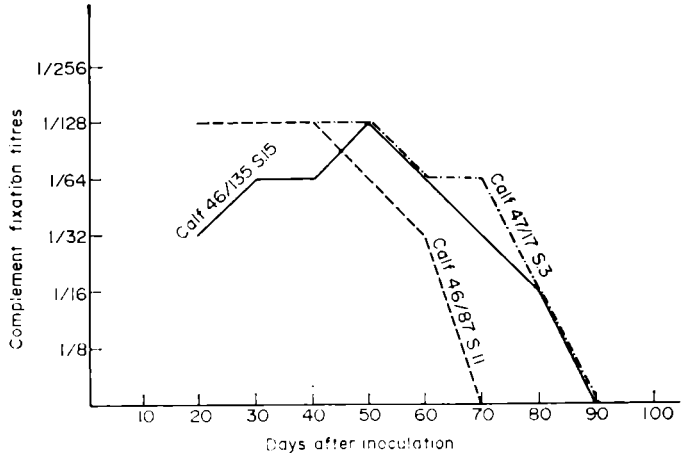
RESULTS

Specificity of the complement fixation test in theileriasis

Sera of 50 cattle which had been shown, by microscope examination, to be free from *T. annulata,* and sera collected at regular intervals from calf No. 47/13 which had been infected with *Anaplasma marginale* and *T. mutans* were submitted to the CF test, using *T. annulata* antigen. None of the sera showed complement fixation. Sera of 142 calves collected at varying intervals after in-

oculation with *T. annulata,* fixed complement in the presence of the *T. annulata* antigen when tested by the complement fixation test.

Serological responses of calves vaccinated with tissue culture vaccine or inoculated with a virulent strain (*Fig.* 1).



The antibody variation in calves inoculated with different strains of *T. annulata* is shown in Fig. 1. Calf 46/135 had been inoculated with S15 (TC vaccine); detectable antibody at 1/32 titre appeared on day 20, reached a maximum of 1/128 on day 50, then gradually decreased and disappeared on the 90th day. Calf 46/87 had been inoculated with S11 (tissue-culture grown schizonts); detectable antibodies appeared at maximum titre on day 20 and persisted up to 40 days post-inoculation. The titre then gradually decreased and disappeared by day 70. Calf 47/17 had been inoculated with S3 (blood); the maximum CF titre, 1/128, appeared on day 40 and persisted to day 50, then gradually decreased and disappeared by day 90. Table I shows the results of complement fixation tests on different sera, obtained from calves inoculated with different strains of *T. annulata* and tested against antigen S15.

The most suitable day to obtain serum from vaccinated animals for complement fixation test

Tests were carried out in order to find out on which day to bleed the vaccinated animals for collecting sera. The results are illustrated in Table II; 100 per cent positive results were obtained on day 40 post-vaccination, the percentage decreasing gradually to 13.6 on day 100.

TABLE I

SEROLOGICAL RESPONSES (CF TITRES) OF CALVES INOCULATED WITH DIFFERENT STRAINS OF *T. annulata* TESTED BY COMPLEMENT-FIXATION AGAINST ANTIGEN S15

Animal No.	*Strain*	*Days after inoculation*									
		10	*20*	*30*	*40*	*50*	*60*	*70*	*80*	*90*	*100*
46/87	11	0	128	128	128	64	16	8	8	0	0
47/80	11	0	32	32	16	16	16	8	0	0	0
46/90	11	0	32	32	32	32	16	16	8	0	0
46/80	11	0	16	128	64	32	16	16	8	0	0
46/81	11	0	16	64	32	16	8	0	0	0	0
46/89	11	0	0	8	8	8	0	0	0	0	0
46/91	11	0	0	0	16	8	8	0	0	0	0
46/92	11	0	0	64	32	16	16	8	8	0	0
46/93	11	0	0	64	32	16	8	0	0	0	0
46/94	11	0	0	32	32	16	16	8	0	0	0
46/95	11	0	0	32	32	16	16	8	8	0	0
47/84	3	0	0	16	32	16	0	0	0	0	0
46/109	3	0	0	32	32	16	16	0	0	0	0
47/105	3	0	0	8	32	32	32	16	16	8	8
47/17	3	0	0	0	128	128	64	64	16	0	0
47/19	3	0	0	0	8	8	0	0	0	0	0
48/16	3	0	0	8	8	8	0	0	0	0	0
48/22	3	0	0	64	64	8	0	0	0	0	0
47/135	15	0	32	64	64	128	64	32	16	0	0
47/69	15	0	16	32	64	8	8	0	0	0	0
47/132	15	0	16	64	64	32	16	8	0	0	0
47/134	15	0	16	64	64	64	32	16	16	16	8
47/81	15	0	8	32	32	32	16	16	16	8	0
46/110	15	0	0	8	64	64	64	64	64	16	0
47/67	15	0	0	16	32	32	64	32	8	8	8
47/69	15	0	0	32	64	8	8	0	0	0	0
47/136	15	0	0	16	32	16	16	8	0	0	0
47/109	15	0	0	0	8	16	0	0	0	0	0
48/2	15	0	0	16	32	32	16	16	8	0	0

Titres are expressed as reciprocal.

TABLE II

INCIDENCE OF POSITIVE REACTIONS TO THE COMPLEMENT FIXATION TEST IN SERA OF CALVES VACCINATED WITH *T. annulata*

Days after vaccination	*Number of sera tested*	*Positive*		*CF titres*					*Negative number*
		number	*percentage*	*1/8*	*1/16*	*1/32*	*1/64*	*1/128*	
10	22	0	0	0	0	0	0	0	22
20	35	11	31	2	4	3	0	1	24
30	32	26	81	4	4	9	7	2	6
40	33	33	100	5	4	15	8	1	0
50	23	22	95·6	5	4	9	2	2	1
60	22	16	77·27	3	5	4	4	0	6
70	22	13	59	2	6	3	2	0	9
80	22	11	50	3	6	1	1	0	11
90	22	7	31·8	5	2	0	0	0	15
100	22	3	13·6	3	0	0	0	0	19

Complement fixation test of sera using homologous and heterologous antigen

A complement fixation test was performed on sera obtained from calf 46/135 using S15 (homologous) and S17 (heterologous) antigens. Sera obtained from calf 48/70, which had been inoculated with S17, were tested against S17 (homologous) and S15 (heterologous) antigens. The serum titres obtained were identical both with heterologous and homologous antigens.

Serological responses of calves vaccinated with tissue culture vaccine and challenged with a virulent strain

Although re-infection of vaccinated animals causes only very mild or even inapparent reactions, in most cases the CF titres are boosted to rather higher levels than the first vaccine reaction. As Table III shows, the figures obtained in the second infection of calf 47/30, which had a maximum titre of 1/64 after vaccine reaction, increased to a titre of 1/256 on day 40 after challenge inoculation. Calf 46/135 had a maximum titre of 1/128 on day 50 post-vaccination, but dropped to 1/64 on day 60. This calf had a maximum titre of 1/128 up to day 100 post-challenge; and still had a titre of 1/16 on day 132.

TABLE III

COMPLEMENT FIXATION TITRES OF VACCINATED CALVES AFTER BEING CHALLENGED WITH *T. annulata* STRAIN S3

Animal no.	*Max. CF titre after vaccination*	*CF titre prior to challenge*	*Days after challenge*								
			20	*30*	*40*	*50*	*60*	*70*	*80*	*90*	*100*
46/97	32	0	64	128	64	32	16	0	0	0	0
46/110	32	0	0	16	8	0	0	0	0	0	0
47/3	64	0	0	64	128	64	64	64	64	32	32
47/1	32	0	0	0	8	16	16	16	16	16	16
47/31	64	0	32	128	32	16	16	16	16	16	16
47/30	64	0	0	128	256	128	64	64	64	64	32
47/67	64	0	16	8	8	0	0	0	0	0	0
47/69	64	0	0	128	128	128	128	64	32	32	16
47/81	64	0	8	64	32	32	8	0	0	0	0
47/132	64	0	0	32	16	0	0	0	0	0	0
47/125	128	0	0	128	128	128	128	128	128	128	128
47/136	32	0	0	16	16	0	0	0	0	0	0
48/2	32	0	0	128	128	64	64	64	32	32	32
47/134	32	0	0	64	32	0	0	0	0	0	0

Titres are expressed as reciprocals.

Complement fixation test of the sera of calves revaccinated one year after the first vaccination

Calves 46/105 and 47/109 were inoculated with the vaccine one year after the first vaccination and their sera were collected regularly. No serological changes due to the second vaccination were observed.

DISCUSSION

A complement fixation test for detecting immunity in cattle recovering from theileriasis has been reported by Konyukhov & Poluboyarova (1967). The antigen used in their complement fixation test was erythrocytic forms of the *T. annulata* parasite and it was successful in detecting complement fixing antibody for at least nine months after the onset of disease. According to these authors resistance to re-infection is correlated with the presence of complement fixing antibodies. In the present experiments the maximum period during which complement fixing antibodies could be detected following first infection was 100 days, and for a longer period following re-infection. The detection of complement fixing antibodies in these experiments was accomplished by using tissue-culture grown schizonts as the antigen. Tissue-culture *T. annulata* vaccine does not produce the erythrocytic forms of the parasite in inoculated animals. Schizonts can be easily produced in tissue culture and freeze-drying of the antigen would allow preparation of a large amount of standardized antigen to be used throughout the experiments and, later, in the routine work.

Calves vaccinated against *T. annulata* will not respond to revaccination with the same strain and do not produce circulating antibody detectable by the complement fixation test. Re-inoculation of vaccinated calves with a heterologous virulent strain will lead to re-appearance of complement fixing antibodies without causing severe clinical reaction. The level of antibody will be boosted to a higher degree than that following the first reaction and can be detected for a longer period. Thus it can be inferred that appearance of complement fixing antibodies is due to multiplication of the parasite in the animal's body and the resistance of animals to homologous re-inoculation is dependent on cellular immunity rather than circulating antibodies.

Neitz (1957) has described the existence of a common antigen in *T. annulata* strains and this is supported by our finding complement fixing antibodies in sera with both homologous and heterologous antigens.

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Anticorps fixant le complément chez le bétail infecté expérimentalement par *Theileria annulata* ou vaccinés avec des cultures de tissus

(Hooshmand-Rad et Hashemi-Fesharki)

Résumé. On a appliqué le test de fixation du complément à la détection d'anticorps fixant le complément dans les sérums de veaux vaccinés contre *Theileria annulata* ou inoculés expérimentalement avec des souches virulentes. L'antigène employé dans la fixation du complément était des schizontes poussés en culture de tissus et lyophilisés. On a démontré l'incidence et la persistance d'anticorps fixant le complément dans les sérums de veaux après vaccination ou après inoculation avec des souches sauvages. On a suggéré quel était le jour le plus favorable pour collecter les sérums des animaux vaccinés en vue de faire le test de fixation du complément.