Comparison of two anticoagulants for production of antigens of *Cowdria ruminantium* in neutrophils

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AWA (D.N.), PAXTON (E.A.), SUMPTION (K.J.) Comparaison de deux anticoagulants pour la production de l'antigène de *Cowdria ruminantium* dans une culture de neutrophiles. *Revue Élev. Méd. vét. Pays trop.*, 1995, **48** (1) : 41-43

L'activité de deux anticoagulants, l'éthylène diamine tétra-acétate (EDTA) et l'héparine, a été comparée sur du sang de chèvres cliniquement infectées par *Cowdria ruminantium*. Les neutrophiles ont été extraits du sang et mis en culture pour la production de l'antigène correspondant. L'EDTA s'est révélé supérieur à l'héparine du point de vue du taux de récupération et d'une meilleure séparation des neutrophiles par rapport aux autres leucocytes. L'antigène produit a été testé avec le test d'immunofluorescence indirecte (IFAT). Il est apparu de bonne qualité. La production de l'antigène par cette méthode est recommandée pour les laboratoire relativement bien équipés dans les zones où la cowdriose est endémique.

Mots clés : Caprin - Cowdriose - *Cowdria ruminantium* - Antigène - Anticoagulant - Granulocyte - Infection expérimentale.

Introduction

The indirect fluorescent antibody test (IFAT) was the first successful serological test for heartwater, developed by Du Plessis (3) using mouse peritoneal macrophages infected with the Kümm strain of *Cowdria ruminantium* as antigen. The enzyme-linked immunosorbent assay (ELISA) test has also recently been used in heartwater serology (4). The current sources of antigen used in IFAT are *C. ruminantium*-infected mouse peritoneal macrophages, neutrophils and endothelial cells (3, 5, 8). The aim of this study was to determine a suitable method of producing the neutrophil antigen, with emphasis on the comparison between the use of EDTA and heparin as anticoagulants for blood intended for cultures.

Materials and Methodi

Experimental infections

Three British Saanen goats numbered G413, G426 and G429 were experimentally infected with the Welgevonden strain of *C. ruminantium*. On the first day of temperature reaction of each goat, 35 ml of blood were collected into EDTA (final concentration 4mM) and in heparin (final concentration 14.3 units/ml) for neutrophil separation and culture. Sampling was continued in goat G426 through days two and three of temperature reaction. Pre-infection (negative) and post-recovery (immune) sera were obtained from a fourth goat which was infected with the same parasite strain but recovered following treatment. The sera were used for testing the antigen.

Neutrophil separation and culture

Blood collected in the two anti-coagulants was given parallel treatment for neutrophil separation and culture. The procedure employed was a modification of the methods described by Logan (6) and Jongejan *et al.* (5). The blood was centrifuged in 10 ml collection tubes at 1000xg for 15 min. The plasma, buffy coat and top layer of the red cell fraction were carefully aspirated and discarded. To the resulting red cell suspension containing the granulocytes, 2 ml of PBSA (phosphate buffered saline pH 7.4, containing 200 µg of streptomycin/ml and 5 µg Fungizone®/ml) (1) was added to every 10 ml original blood and mixed thoroughly. An equal volume of distilled water was added to the resultant suspension and mixed for 30 seconds to lyse the red cells. Isotonicity was restored by adding 6 ml of 2.7 % NaCl. The resultant solution was centrifuged at 200xg for 10 min to pellet the granulocytes. The pellet was resuspended in 10 ml sucrose phosphate glutamate buffer and washed twice for 8 min at 200xg. The final pellet was resuspended in RPMI 1 640 culture medium (Gibco Ltd, Paisley Scotland), containing 10 % inactivated foetal calf serum, 2mM L-glutamine (Gibco), 100 units of penicillin and 100 µg of streptomycin/ml, to give a count of 1-2 x 10^6 cells/ml and incubated at 37°C in a humidified incubator gassed with 5 % CO2 in air. Cytospin smears were prepared from the culture by spinning about 5 µl per well in a cytocentrifuge (Shandon Cytospin 2) at 1000 rpm for 5 min. On the day of culture preparation, the smears were stained with Giemsa and examined for purity (differential count of leucocytes) and the presence of *Cowdria*. This continued on days one, two and three of culturing to determine the best time to prepare smears for antigen, judging from the appearance of *Cowdria* in neutrophils. Once this was decided, smears were prepared, air-dried and fixed in acetone for 10 min, wrapped in aluminium foil, put in polythene bags containing silica gel and stored at -20°C until use.

Indirect fluorescent antibody test

The antigen was tested in the indirect fluorescent antibody test (IFAT). The slides were removed from -20°C and allowed to thaw at room temperature in a moist chamber. Serial dilutions from 1:8 to 1:10000 were made from the negative and the immune sera using phosphate buffered saline (PBS) as diluent. Wells were made around the cytospin smears on the slides with a ball point paint marker (Texpen, Merck Ltd). Sera were applied to the wells and the slides were incubated in a moist chamber at room temperature for 30 min. The slides were then washed three times in PBS for a total of 15 min. Rabbit anti-sheep-and-

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goat globulins conjugated with fluorescein isothiocyanate containing 2 % Evans Blue counter stain (Nordic Immunology, the Netherlands) was then applied to the wells and the slides were again incubated for 30 min, then washed in PBS as before. They were then mounted in 90 % glycerol in FA buffer (pH 7.5 and made up of 11.5 g Na_2HPO_4, 2.96 g NaH_2PO_4, and 5.84 g NaCl per litre of distilled water) under a cover slip and examined at x40 using a fluorescent microscope (Laborlux K, Leica UK Ltd).

Results

The results of neutrophil separation are presented in table I. EDTA proved to be superior to heparin both in the recovery rate of neutrophils and the purity of the culture. Purer cultures were obtained when the upper red cell layer was removed. These values were particularly high in EDTA separations ranging from 93 to 99 % compared to heparin with values ranging from 14 to 74 % in equivalent samples. Due to the low neutrophil recovery rate with heparin as anticoagulant, cultures for antigen production were only made from EDTA samples. On the day of collection, no *Cowdria* organisms could be demonstrated in cytospin smears of separated neutrophils, but organisms started appearing after 24 h of culture. The highest percentage and optimal rate of infected cells was seen after 48 h of culture and this was when antigen slides were prepared. On days three and four of culture, the organisms became less discernable because of the degeneration of the neutrophils.

IFAT gave good specific fluorescence with dilutions of 1:64 to 1:10000. The morphology of fluorescing *Cowdria* morulae was comparable to that seen in Giemsa-stained cytospin smears. Background non-specific fluorescence was easily distinguishable since it was weak and diffuse. All dilutions of the negative serum gave only background fluorescence.

Discussion

The technique for culture of *Cowdria ruminantium* in neutrophils was first developed by Logan *et al.* (7). Jongejan *et al.* (5) used the technique for the production of antigen for use in IFAT. The quantity and quality of antigen produced depends on how well the neutrophils are separated from the rest of the blood. The above authors used heparin as anti-coagulant; however, in preliminary studies we found that separation of neutrophils from lymphocytes was poor and the recovery rate low from blood containing heparin as anticoagulant. This prompted the comparative use of EDTA as anti-coagulant which proved superior in both aspects. The quality of antigen slides produced was good, indicating that EDTA had no observable adverse effect on the development of *Cowdria* in neutrophils.

Carlson and Kaneko (2) obtained similar results for the separation of neutrophils from bovine blood. Their recovery rate with EDTA as anticoagulant was 63.9 %. Although 65.6 % recovery was achieved in one sample in the present study, recovery rate was generally lower with an average of 37.7 %. Carlson and Kaneko (2) may have achieved a higher average because they also extracted neutrophils from the buffy coat which was discarded in the present study. In heparinised blood samples, the buffy coat was poorly formed, accounting for the poor separation and low recovery of neutrophils. In this study, the recovery rate of neutrophils was between 1.6 and 38 times higher from blood collected into EDTA than with heparin, and therefore a much more efficient production of antigen slides can be made through the use of no greater volume of culture medium or reagents.

Conclusion

The culture of neutrophils from the blood of goats reacting to *Cowdria ruminantium* infection for antigen production proved to be very successful. The technique is

TABLE I
Results of neutrophil separation with EDTA and heparin

Animal	Days post onset Fever	Anticoagulant (E, EDTA ; H, Heparin)	Blood Neutrophils Count ($\times 10^6$/ml)	Neutrophils Recovered ($\times 10^6$/ml)	(% of blood count)	% Neutrophils after extraction (Neutrophil count/ leucocyte count)
G413	1	E	3.05	2	(66)	66
		H	3.05	1.28	(42)	44
G426	1	E	5.93	1.8	(31)	97
		H	5.93	0.07	(1.2)	14
G426	2	E	5.0	2.3	(46)	93
		H	5.0	0.06	(1.2)	65
G426	3	E	6.6	1.4	(21.2)	99
		H	6.6	0.1	(1.5)	74
G429	1	E	6.5	1.6	(24.6)	59
		H	6.5	0.09	(1.4)	14

simple and can easily be carried out in moderately well-equipped laboratories in heartwater-endemic areas where serological surveys may be required. EDTA is therefore recommended as anticoagulant for blood collection intended for neutrophil culture because of the higher recovery rate. The use of this anticoagulant should result in more efficient and economical production of antigen slides for sero-diagnosis, with the use of fewer infected animals. This should facilitate antigen production in developing countries.

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Ethylene diamine tetra-acetate (EDTA) and lithium heparin were used comparatively as anticoagulants for blood obtained from goats clinically infected with *Cowdria ruminantium*. Neutrophils were extracted from the blood and cultured for the production of heartwater antigen. EDTA proved superior to heparin in terms of the recovery rate and the better separation of neutrophils from other leucocytes. The antigen produced was tested in the indirect fluorescent antibody test (IFAT) and proved to be of good quality. Production of antigen slides by this method is recommended for moderately well-equipped laboratories in heartwater-endemic areas.

Key words: Goat - Heartwater - *Cowdria ruminantium* - Antigen - Anticoagulant - Granulocyte - Experimental infection.