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ISOLATION OF *COWDRIA RUMINANTIUM* BY MEANS OF PERCOLL DENSITY GRADIENT CENTRIFUGATION AND DETECTION BY AN ENZYME-LINKED IMMUNOSORBENT ASSAY

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ABSTRACT

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The isolation of *Cowdria ruminantium* by means of Percoll density gradient centrifugation permits the recovery of partially purified viable populations of the organism possessing distinctly different densities. These conclusions are based upon results of analyses of density fractions by intravenous inoculation into sheep, protein determination, electron microscopy and enzyme-linked immunosorbent assay. Morphological differences were observed in the density fractions obtained from infected brain tissue and *Amblyomma hebraeum* nymphae.

INTRODUCTION

Density gradient centrifugation has been employed for large scale isolation of cells, subcellular particles and viruses under mild conditions (Anderson, 1966).

Various density gradient media for the purification of rickettsial organisms have been reported. These include Renografin (Howard, Orenstein & King, 1974), sucrose (Wang & Grayston, 1967) or Percoll (Tamura, Urakami & Tsuruhara, 1982). The use of Percoll offers numerous advantages and has been applied to the isolation of a variety of cells with complete retention of morphological integrity and biological activity (Schumacher, Schäfer, Holstein & Hilz, 1978).

Cowdria ruminantium, the causative agent of heartwater (Cowdry, 1925), has been shown to vary extensively in size and to be extremely pleomorphic (Pienaar, 1970; Uilenberg, 1983). A method, involving cellular affinity chromatography (Viljoen, Vermeulen, Oberem, Prozesky, Verschoor, Bezuidenhout, Putterill, Visser & Neitz, 1985) for the isolation of *C. ruminantium* was found to be suitable for the preparation of sufficient quantities of partially purified heartwater organisms needed as antigen in an ELISA. This method is unsuitable, however, for descriminating between the various sizes and forms of the organism. The present paper describes the application of Percoll as density gradient medium for the isolation of *C. ruminantium* and for the separation of a heterogeneous population of this organism. Through such separations, morphological and biochemical investigations of the organism should be facilitated. Infected brain tissue and *Amblyomma hebraeum* nymphae were used as starting material. An ELISA and electron microscopy were employed for the identification of the organism.

MATERIALS AND METHODS

Analytical quality reagents were used in all the experiments. Glassware and equipment were sterilised with 70 % (v/v) ethanol and the buffers by filtration through 0,22 μm filters[(1)].

Preparation of crude brain and nymph extracts

Sheep brain and *A. hebraeum*, Spes Bona strain nymphae, were infected with Onderstepoort Ball 3 strain of *C. ruminantium* (Viljoen, *et al.* 1985).

Heartwater infected and non-infected sheep brain tissue (120 g to 250 g wet frozen mass) were preserved in liquid nitrogen. When required, the tissue was quickly thawed and homogenized at low speed in a Waring Blender[(1)] for 5 min in 60 to 125 mℓ of 0,154 M NaCl. All subsequent isolation steps were done at 4 °C.

Infected and non-infected *A. hebraeum* nymphae were homogenized for 10 min in 0,154 M NaCl with an Ultra Turrax[(2)] at a dilution of 10 nymphae per 5 mℓ saline.

The brain and tick homogenates were centrifuged for 30 min at 1 000 × g in a Rotor 19 using a Beckman L5-65 ultracentrifuge. The supernatant was then centrifuged for 30 min at 10 000 × g with a Rotor 30 and the resultant supernatant centrifuged at 30 000 × g for 30 min. The final sediment was resuspended in 0,5 mℓ of 0,154 M NaCl. This represented the crude extract.

Percoll density gradient centrifugation

An iso-osmotic Percoll stock solution (SIP) with density of 1,123 g/mℓ was prepared by adding 9 mℓ of Percoll[(3)] to 1 mℓ of 1,54 M NaCl.

The resuspended crude extracts (0,5 mℓ) were then layered on top of the gradient forming material prepared by mixing 15 mℓ SIP with 14,5 mℓ of 0,154 M NaCl to form a 50 % density SIP solution. As reference, a tube containing 50 % density SIP solution and density marker beads[(4)] was used.

Centrifugation was performed for 15 min at 30 000 × g in a Rotor 30 employing maximal acceleration and half maximal braking. Hereafter the gradient was collected from the bottom of the tube with a fraction recovery system[(5)]. Ten fractions of 3 mℓ each were collected and used immediately or stored at −30 °C.

Determination of infectivity

Sheep of *c.* 40 kg body mass of either sex were injected intravenously at a dosage rate of 2 mℓ per animal with the crude extracts and 2,5 mℓ per animal with the Percoll fractions. The injection needle was dipped into a

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(1) Millipore, South Africa, (Pty) Ltd

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(1) Waring Products Division, U.S.A.
(2) Janke & Kunkel, Kika-Werk, Fed. Rep. Germany
(3) Pharmacia Fine Chemicals, Uppsala, Sweden
(4) Pharmacia Fine Chemicals, Uppsala, Sweden
(5) Beckman Instruments, Palo Alto, U.S.A.

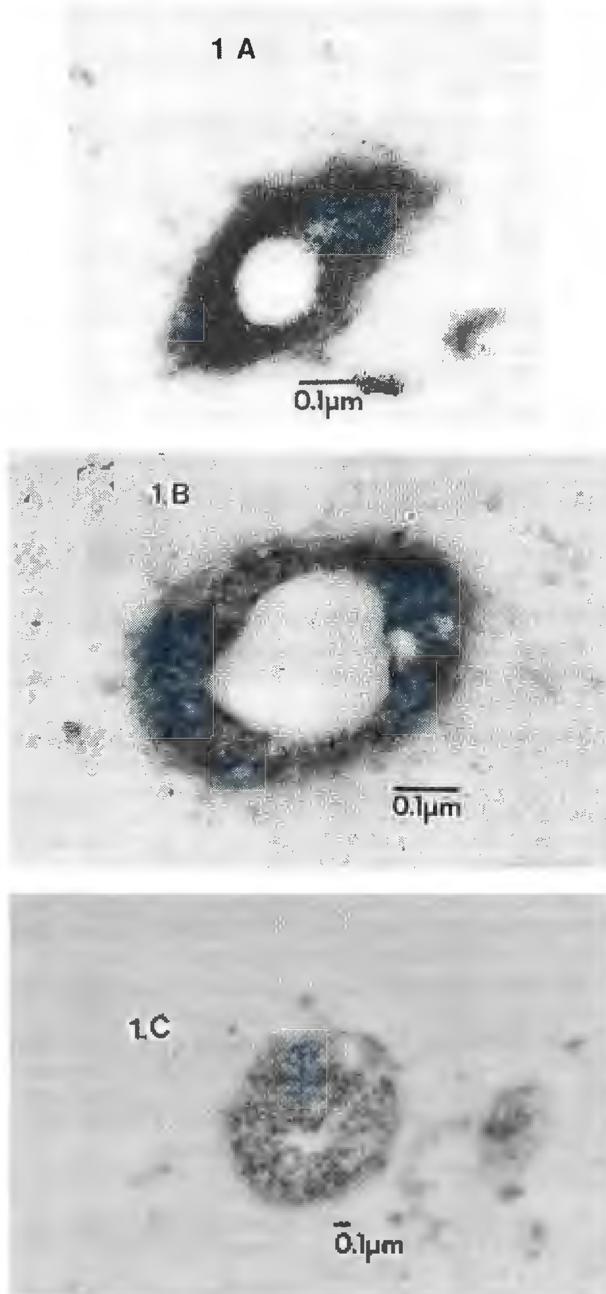

FIG. 1 Electron micrographs of suspected *Cowdria ruminantium* organisms isolated on a Percoll density gradient starting with heartwater infected sheep brain material; 1A—fraction 2 (1,109 ± 0,017 g/cm³), × 80 000; 1B—fraction 5 (1,058 ± 0,001 g/cm³), × 100 000; 1C—fraction 8 (1,050 ± 0,002 g/cm³), × 70 000. Bar scale = 0,1 µm.

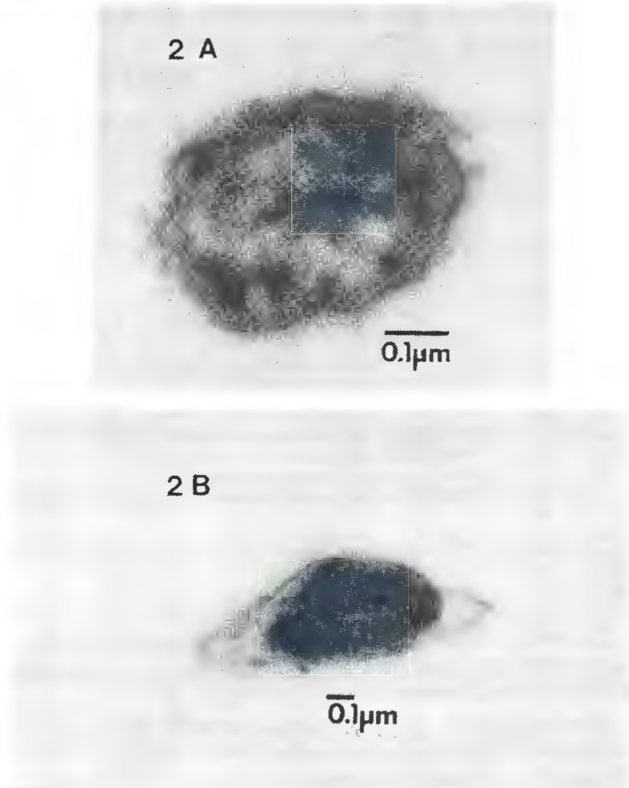

FIG. 2 Electron micrographs of suspected *Cowdria ruminantium* organisms isolated on a Percoll density gradient starting with heartwater infected *Amblyomma hebraeum* nymphae; 2A—fraction 2 (1,058 ± 0,001 g/cm³), × 100 000; 2B—fraction 8 (1,050 ± 0,002 g/cm³), × 80 000. Bar scale = 0,1 µm.

1 % adrenalin solution[1] prior to the injection of the crude extracts to prevent anaphylactic shock. Rectal temperatures were taken daily and the animals were kept under observation for at least 21 days. If no rise in temperature occurred, the sheep were challenged 21 days after the initial inoculation with 5 mℓ (1 dose) Onderstepoort heartwater Ball 3 vaccine. Reacting sheep were allowed to die after which a necropsy was performed. Positive diagnosis of heartwater was made after microscopic detection of *C. ruminantium* colonies in Giemsa stained brain smears (Pienaar, 1970; Purchase, 1945).

Protein determinations

The protein content of the density gradient fractions were determined according to a modification of the Lowry method (Maddy, 1976). The Folin-Ciocalteau[1] reagent was diluted 1:1 with distilled water. Colour development was monitored at 660 nm on a Beckman Model 25 spectrophotometer against a blank, consisting of the corresponding density fraction obtained from the control run. In some cases the results obtained were compared to those from the high temperature Biuret-Folin method described by Dorsey, McDonald & Roels (1977).

Electron microscopic studies

Aliquots (0,5 mℓ) of crude extracts as well as of the density gradient fractions were made up to 10 mℓ with 0,154 M NaCl and centrifuged for 30 min at 30 000 × g in a Rotor 40 to sediment the organisms. The supernatants were removed and the sediment of each fraction was mixed with *c*. 0,1 mℓ of 2 % (w/v) agar at 45 °C and siphoned into a glass capillary tube. The content of the tube was streaked across filter paper, which was then cut into narrow strips with a Swan-Morton Scalpel. The preparation and embedding of these sections were performed as described by Glauert (1965). The sections were embedded in Spurrs[2] solution and thin sections (0,1 µm) were cut with a Reichert-Jung Ultracut Microtome[3]. These were stained for 45 min in a 4 % aqueous solution of uranyl acetate and for 10 min in lead citrate, at room temperature and on G300 copper grids[4]. The grids were investigated with a Hitachi H600 electron microscope[5].

Enzyme-linked immunosorbent assay (ELISA)

A modification of the ELISA method described by Notermans, Timmermans & Nagel (1982) was used.

[1] Centaur, South Africa

[1] Merck, Darmstadt, Fed. Rep. Germany
[2] Polaron Equipment Ltd, U.S.A.
[3] Reichert-Jung, Austria
[4] Polaron, Micro-structure (Pty) Ltd, U.S.A.
[5] Hitachi, Japan

TABLE 1 The density, protein content and electron microscopic appearance of heartwater infected and non-infected brain or nymph material after Percoll density gradient centrifugation

Percoll fractions	Density g/cm³	Brain material				Nymph material			
		Protein content (mg)		Electron microscopy		Protein content (mg)		Electron microscopy	
		I[1]	N[2]	I	N	I	N	I	N
Crude extract		9,0 ± 0,2	1,5 ± 0,15	+[3]	–[4]	8,0 ± 0,3	0,7 ± 0,10	+	–
1	1,134 ± 0,008	1,3 ± 0,1	0,08 ± 0,02	+	–	0,9 ± 0,1	0,02 ± 0,01	+	–
2	1,109 ± 0,017	0,2 ± 0,1	0,06 ± 0,03	+	–	0,3 ± 0,1	0,01 ± 0,01	+	–
3	1,078 ± 0,013	0,7 ± 0,15	0,09 ± 0,03	+	–	0,4 ± 0,05	0,02 ± 0,01	+	–
4	1,062 ± 0,003	0,4 ± 0,05	0,07 ± 0,03	+	–	0,4 ± 0,03	0,03 ± 0,01	+	–
5	1,058 ± 0,001	1,1 ± 0,1	0,10 ± 0,03	+	–	0,9 ± 0,05	0,08 ± 0,015	+	–
6	1,056 ± 0,001	1,2 ± 0,2	0,11 ± 0,04	+	–	1,2 ± 0,1	0,1 ± 0,01	+	–
7	1,054 ± 0,002	0,6 ± 0,15	0,05 ± 0,03	+	–	0,7 ± 0,02	0,05 ± 0,01	+	–
8	1,050 ± 0,002	0,5 ± 0,2	0,04 ± 0,03	+	–	0,6 ± 0,03	0,02 ± 0,01	+	–
9	1,045 ± 0,004	1,4 ± 0,15	0,28 ± 0,04	+	–	1,1 ± 0,1	0,1 ± 0,02	+	–
10	1,034 ± 0,007	1,5 ± 0,2	0,42 ± 0,04	+	–	1,3 ± 0,2	0,2 ± 0,03	+	–

[1] I — Heartwater infected
[2] N — Heartwater non-infected
[3] + — Presence of suspected heartwater organisms
[4] – — No suspected heartwater organisms

TABLE 2 Infectivity in sheep of fractions obtained after Percoll density gradient centrifugation

Percoll fractions	Density g/cm³	Brain material		*Amblyomma hebraeum* Spes Bona strain		
		I	II	III	IV	V[2]
Crude extract		+ (14)[1]	+ (17)	A	A	A
1	1,134 ± 0,008	+ (12)	+ (11)	+ (11)	+ (18)	+ (10)
2	1,109 ± 0,017	+ (11)	+ (10)	+ (8)	+ (18)	+ (8)
3	1,078 ± 0,013	+ (13)	+ (11)	+ (8)	+ (17)	+ (7)
4	1,062 ± 0,003	–	+ (12)	+ (10)	–	+ (7)
5	1,058 ± 0,001	–	+ (10)	+ (10)	–	+ (8)
6	1,056 ± 0,001	–	+ (11)	+ (8)	–	+ (9)
7	1,054 ± 0,002	–	+ (12)	+ (8)	–	+ (8)
8	1,050 ± 0,002	+ (13)	+ (12)	+ (11)	–	+ (9)
9	1,045 ± 0,004	+ (10)	+ (10)	0	+ (17)	+ (8)
10	1,034 ± 0,007	+ (11)	+ (10)	0	–	+ (8)

[1] The day on which the first heartwater symptoms (constant high rectal temperature) appeared, is given in parenthesis
[2] I to V — Experiment number
+ — Positive reaction (raised temperature)
– — No reaction in sheep (temperature normal during incubation period)
A — Not biologically tested
0 — Sheep died *c.* 15 min after injection, probably as a result of anaphylactic shock

Sheep brain crude extracts, nymphae crude extracts as well as density gradient fractions were screened for antigenic properties.

Antisera from heartwater infected sheep were prepared as follows: Blood samples (10 mℓ) were left to clot and after 2 hours the coagulated blood was centrifuged for 10 min at 300 × g in a Piccolo bench top centrifuge at room temperature. The serum was siphoned off, divided into 1 mℓ batches and stored at –30 °C. A serum solution was made up as follows: 1 mℓ was diluted 1:30 with a 0,05 M Tris, 0,1 M NaCl, buffer pH 7. Optimal serum and antigen dilutions were selected as described by Viljoen *et al.* (1985). Antigen was sonified for 5 seconds with a Sonifier Cell Disruptor B-30[1], using a micro tip at 40 % continuous duty cycles and a setting of 3 on the output control.

Microtiter[2] plate wells were coated with 100 $\mu\ell$ antigen (Ag) at a concentration of 10 μg protein per mℓ of the Tris buffer. The plates were incubated for 2 h and subsequently washed twice with 0,05 M Tris, 0,1 M NaCl, 0,05 % Tween 20 (v/v), pH 7 and 3 times with distilled water. All incubations were done at room temperature while gently shaking the plate on a mechanical plate shaker[2]. The washing solution was siphoned off after each wash. The blocking buffer, 3 % bovine serum albumin (BSA) (w/v) in Dulbecco's phosphate buffered saline (PBS), was applied in 200 $\mu\ell$ quantities to block all vacant sites. After incubation for 1 h, the blocking buffer was siphoned off. The serum, containing 0,1 mg protein in 25 mℓ of 0,05 M Tris, 0,1 M NaCl, pH 7, was then added to the microtiter plate in 100 $\mu\ell$ quantities and incubated for 1 h. The plate was washed with 500 mℓ of 0,05 % (v/v) Tween 20 in PBS and once with 500 mℓ of 1 % BSA (w/v) in PBS. A stock solution of 0,5 mg Protein A-alkaline phosphatase[1], in 50 mℓ distilled water was prepared. Of this solution, 5 $\mu\ell$ was diluted to 7,5 mℓ with 1 % BSA (w/v) in PBS, and 50 $\mu\ell$ was added to each well. After incubation for 40 min the plate was washed 6 times with 100 $\mu\ell$ per well of a 1 % BSA (w/v) PBS solution.

To 10 mℓ of the freshly made substrate buffer, containing 3,16 g of 2-amino-2-methyl-1:3-propanediol and 40,6 mg $MgCl_2.6H_2O$ in 100 mℓ distilled water, 35 mg p-nitro phenylphosphate was added. Of this solution, 50 $\mu\ell$ was added to each well and the colour development was monitored immediately for 120 min with a Titertek

[1] Branson Sonic Power Company, Danbury, U.S.A.
[2] Linbro Division, Flow Laboratories, McLean, Virginia, U.S.A.

[1] Sigma, St Louis, U.S.A.
[2] Linbro Division, Flow Laboratories, McLean, Virginia, U.S.A.

Multiscan MC[2] at 690 nm and 405 nm. Signal to background ratios were calculated from absorbancies obtained for infected and corresponding non-infected fractions.

RESULTS

All infected crude brain extracts were infective after i.v. inoculation (Table 2). After Percoll density gradient centrifugation of crude heartwater suspensions, *C. ruminantium* organisms and antigen were dispersed widely in the gradient according to biological tests (Table 2) and ELISA methods (Table 3). Suspected *C. ruminantium* organisms were seen in all density fractions according to electron microscopic investigations (Fig. 1 & 2 and Table 1). In non-infected brain material very little or no background was observed electron microscopically. In the case of non-infected nymph material, unidentified rickettsial organisms other than *C. ruminantium* were observed. Although a limited number of organisms were studied, it appeared that different populations of heartwater organisms were present at different densities; the smaller ones (0,45 ± 0,15 μm) at a density of 1,134 g cm^{-3} and the larger (1,00 ± 0,2 μm) at a density of 1,034 g cm^{-3}. An increase in organism size was observed with a decrease in density. No significant ultrastructural differences were observed between *C. ruminantium*-like organisms from brain and nymph material. The only morphological difference noticed was the presence of a single defined electron-transparent region in the electron micrograph of organisms obtained from brain tissue (Fig. 1), whereas organisms from nymph material displayed several electron-transparent areas (Fig. 2).

TABLE 3 ELISA detection of heartwater organisms after Percoll density gradient centrifugation: Ratios[1] of absorbance values obtained with infected and non-infected material as antigen

Percoll fraction	Density g/cm³	Brain material R[2]	Nymph material R[2]
Crude extracts		1,53 ± 0,6	1,82 ± 0,5
1	1,134 ± 0,008	1,36 ± 0,2	1,48 ± 0,2
2	1,109 ± 0,017	2,30 ± 0,3	2,72 ± 0,5
3	1,078 ± 0,013	1,54 ± 0,4	1,84 ± 0,5
4	1,062 ± 0,003	1,70 ± 0,3	1,61 ± 0,4
5	1,058 ± 0,001	2,21 ± 0,5	2,36 ± 0,4
6	1,056 ± 0,001	1,1 ± 0,2	1,88 ± 0,5
7	1,054 ± 0,002	1,1 ± 0,1	1,55 ± 0,2
8	1,050 ± 0,002	2,1 ± 0,4	2,54 ± 0,4
9	1,045 ± 0,004	1,2 ± 0,4	1,55 ± 0,3
10	1,034 ± 0,007	1,2 ± 0,5	1,42 ± 0,6

[1] Mean values ± standard deviations for 6 replicates
[2] Heartwater infected to heartwater non-infected ratio

With the ELISA, Table 3, the highest signal (infected) to background (non-infected) ratios were observed at densities of 1,109 g cm^{-3}, 1,058 g cm^{-3} and 1,050 g cm^{-3}.

DISCUSSION

It is generally recognized that the purification of *C. ruminantium* is more difficult than the purification of other rickettsiae. This is due to *C. ruminantium* being a particularly labile organism (Uilenberg, 1983).

Infected crude brain extracts were shown to be infective when inoculated i.v. Only on one occasion has this route of injection of brain material been proven to be successful in transmitting heartwater (Uilenberg, 1971). This is contrary to the results of Ilemobade & Blotkamp (1978) who were able to cause infection by infected brain homogenates through the s.c. route only. I.v. inoculations either failed to cause disease or resulted in immediate fatalities. These contradicting findings could possibly be explained by the fact that our crude brain extracts were prepared by differential centrifugation of brain homogenates. This suggests that certain unknown components in brain homogenates may prevent infectivity by the i.v. route.

The presence of pathogenic and viable *C. ruminantium* organisms over a density range from 1,134 g cm^{-3} to 1,034 g cm^{-3} exemplifies the pleomorphism of this organism, but could also be due to the presence of organisms in different stages of development. Similar observations were made for *Rickettsia tsutsugamushi* (Tamura *et al.*, 1982).

The location of a single, defined electron-translucent area observed in electron micrographs of *C. ruminantium*-like organisms from brain has also been reported by Prozesky & Du Plessis (1985). In organisms from nymph material such defined, single electron-pale areas were absent; instead, several scattered electron-pale areas were observed.

The fractions obtained at densities of 1,109; 1,058 and 1,050 g cm^{-3}, had an ELISA signal to noise ratio of more than 2:1 suggesting that the antigenic material was more concentrated at these levels. Protein content peaked at densitites of 1,134; 1,078; 1,058; 1,056; 1,045 and 1,034 g cm^{-3}.

Infectivity incubation periods could not be correlated with ELISA colour development or protein content. Protein determinations showed that the infected fractions had a higher protein content than the non-infected fractions, suggesting that the protein content of these fractions relates to the infected state of the tissue. This observation is based on equal wet frozen mass of brain material and equal amounts of infected and non-infected nymphae. However, a simple relationship could not be demonstrated between organism concentration (protein content), antigenicity (ELISA) and pathogenicity (incubation period).

As expected, electron microscopy of the infected nymph material showed contaminating micro-organisms, probably belonging to the family Rickettsiaceae described by Cowdry (1925). Therefore brain material, in which no contaminating organisms could be detected, is a more suitable source of starting material for the purification of *C. ruminantium*.

The isolation procedure described in this paper represents a quick and mild method by which different cell populations of *C. ruminantium* can be obtained in a partially purified and viable form as demonstrated by electron microscopic studies, ELISA and infectivity assays.

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