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Characterization of Monoclonal and Polyclonal Antibodies to Bovine Enteric Coronavirus: Establishment of an Effecient ELISA for Antigen Detection in Feces

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ABSTRACT

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Monoclonal antibodies to bovine enteric coronavirus (BEC) were produced. Additionally, polyclonal antibodies were made in rabbits and guinea pigs and extracted from the yolk of immunized hens. The antibodies were characterized by neutralization test, hemagglutination inhibition test, enzyme-linked immunosorbent assay (ELISA) and immunoblotting. Neutralizing antibody titers of polyclonal antisera ranged from 1 : 1280 to 1 : 40 000. Only one out of 908 hybridoma colonies tested secreted antibodies with neutralizing activity. By ELISA, polyclonal sera exhibited high background reactions that could be significantly reduced by treatment with kaolin in the case of rabbit sera.

Attempts to establish an ELISA for BEC antigen detection based on polyclonal sera failed due to low sensitivity and specificity. Optimal results were achieved when a mixture of two monoclonal antibodies was coated onto microplates for antigen capture, while rabbit hyperimmune serum served as detecting antibodies in an indirect assay.

The combination of the two monoclonal antibodies did not increase sensitivity synergistically, but in a compensatory fashion, probably because of epitope differences between BEC field strains.

INTRODUCTION

Bovine enteric coronaviruses (BEC) are important pathogens inducing diarrhea in newborn calves worldwide. They are found alone or in association with rotaviruses, enterotoxigenic *Escherichia coli* or cryptosporidia (Baljer and Bachmann, 1980; Reynolds et al., 1986; Snodgrass et al., 1986; Baljer et al., 1987). The detection of BEC in feces still gives much difficulty and requires

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elaborate techniques. The most commonly applied methods for direct virus detection are negative staining electron microscopy (Stair et al., 1972) and immune electron microscopy (Almeida and Waterson, 1969). The unquestionable advantage of very rapid diagnosis is counteracted by the high cost of equipment, low detection limit (10^6 particles ml^{-1}), limited number of samples which can be processed and subjective assessment by the investigator. Immunofluorescent antibody techniques (Mebus et al., 1973; Woode et al., 1978) can only be used for postmortem diagnosis and depend on fresh material, which is not usually available for routine diagnosis. Sharpee et al. (1976) established an assay in which the specificity of the hemagglutinating activity of BEC is confirmed by a hemagglutination inhibition (HI) test. However, neither this test nor the hemadsorption–elution–hemagglutination test (HEHA) as described by Ellens et al. (1978) met with general acceptance in routine diagnosis. Nonspecific hemagglutination (HA) reactions caused by other hemagglutinating agents occurring in fecal samples (e.g., enteroviruses) and the temperature dependence in HA of different BEC strains are responsible for non-reproducible results. The reversed passive hemagglutination assay (RPHA, Sato et al., 1984) can be carried out with impressive ease, but a high percentage of false-positive reactions causes difficulties in practice. Additionally, antisera purified by affinity chromatography have to be used.

Attempts have been made to develop enzyme-linked immunosorbent assays (ELISAs) for the detection of BEC in feces, but only three assay configurations have been reported so far. Two procedures have been devised as blocking ELISAs using the double sandwich technique based on polyclonal antibodies only (Ellens et al., 1978; Reynolds et al., 1984), whereas Crouch et al. (1984) described a test with two monoclonal antibodies for antigen capture and a direct polyclonal detection system. Unfortunately, only a few results are available on the success of these assays in practice.

In this study, various ELISA strategies for coronavirus detection were investigated and compared. An efficient and sensitive assay for routine diagnosis which can be carried out rapidly and easily is described.

MATERIALS AND METHODS

Propagation and purification of BEC

The cell culture-adapted isolates of BEC 800 Holland, P 1239 France, RIT-Genoval Belgium, V270/83 Munich and V410/83 Munich were propagated in primary or secondary bovine fetal lung cells (BEL) with Earle's MEM containing 2% fetal calf serum. Cell culture suspensions of BEC V270/83 Munich were freeze-thawed ($-70/20\,^{\circ}C$) and clarified by centrifugation at $6000 \times g$ for 20 min. The virus was pelleted in a Beckman SW 28-rotor at 28 000 r.p.m. for 60 min. After resuspension in TEN buffer (0.02 M Tris–HCl, 0.15 NaCl, 0.001

M EDTA, pH 7.6), the sediment was used for ELISA (semipurified BEC) or layered onto a pre-formed 20–60% (w/w in TEN buffer) linear sucrose gradient and centrifuged at 30 000 r.p.m. for 180 min in a Beckman SW 60 rotor. The visible virus band was collected by side puncture, pelleted in a Beckman SW 40 rotor at 40 000 r.p.m. for 60 min and resuspended in TEN buffer.

Fecal samples

Fecal samples used in these studies originated from calves (aged 1 to ~50 days) with diarrhea and had been sent to the diagnostic unit of our institute between 1982 and 1986. Most of the farms were located in the southern part of Germany. Upon arrival, the samples were diluted 1:5 in phosphate-buffered saline (PBS), thoroughly suspended in a Lab-Blender (Kleinschmidt, Hannover) and centrifuged at $2200 \times g$ for 30 min. HA titers of the supernatants tested in the antigen ELISA were >1:200 and had been verified as BEC specific by the HI test with antisera from rabbits (kindly supplied by D.J. Ellens). Endpoint titers were not determined.

Polyclonal antisera

Chickens were hyperimmunized i.m. five times at intervals of 4 weeks with 0.2 ml semipurified BEC (HA titer 1:40 000) emulsified in the same volume of complete Freund's adjuvant. Antibodies were extracted from yolks using isopropanol/acetone as described by Bade and Stegemann (1984).

Rabbits and guinea pigs were immunized four times s.c. with 0.4 ml gradient-purified BEC (HA titer 1:40 000). Complete and incomplete Freund's adjuvants were used. The animals were bled by cardiac puncture 14 days after the final booster.

To reduce non-specific reactions in hyperimmune sera, they were inactivated at 56°C for 30 min and treated with kaolin (25% in 0.85% NaCl; Mayr et al., 1974). After incubation for 30 min at room temperature, supernatants were collected by centrifugation at $2200 \times g$ and additionally purified on Protein A–Sepharose.

Monoclonal antibodies (MAb)

Eight-to twelve-week-old BALB/c mice were given three i.p. injections of 0.1 ml purified BEC (HA-titer 1:20 000) at 21-day intervals. For the final booster, 0.05 ml were applied intravenously 3 days before fusion. Spleen cells were mixed with the mouse myeloma cell line P3-X63 Ag 8.653 and fused using 40% PEG 4000 (Merck, Darmstadt) as described by Fazekas de St. Groth and Scheidegger (1980). The cells were suspended in HAT medium and dispensed in 96-well tissue culture plates (Falcon, Becton Dickinson). Supernatants were

screened for antibody production by ELISA, microneutralization test (NT) and HI 2 weeks later. Hybridomas secreting antibodies specific for BEC were cloned by limiting dilution, and serum-free cell culture supernatants as well as BALB/c ascitic fluids were produced.

For the determination of immunoglobulin subclasses, serum-free hybridoma supernatants were diluted 1:2 in carbonate/bicarbonate buffer and coated onto microtiter plates. The immunoglobulin subclass was determined using anti-mouse isotype-specific antisera produced in goats (Sigma, Munich).

IgG was isolated from ascitic fluids by affinity chromatography on Protein A–Sepharose.

Antibody screening tests

NT and HI were performed by standard methods using 100 $TCID_{50}$ per 0.05 ml BEC and 5×10^5 BEL cells ml^{-1}, or 4 HAU and a 0.5% suspension of rat erythrocytes in NaCl–phosphate buffer, respectively. For indirect ELISA, microplates (Immuno II, Nunc, Wiesbaden) were coated with semipurified BEC diluted 1:400 in carbonate/bicarbonate buffer (pH 9.6). Antibodies to be screened were incubated for 60 min at 37°C. Binding was detected by peroxidase-conjugated anti-species IgG globulin and made visible using 3′3′5′5′ tetramethylbenzidine (TMB, Serva, Heidelberg) as indicator. Ten minutes later, the reaction was stopped by the addition of 2 M H_2SO_4 and measured in a Titertek photometer (Flow, Bonn) at 450 nm. Extinctions > 0.1 were considered positive. Between each incubation step, plates were washed four times with NaCl–phosphate buffer containing 0.05% Tween 20. To estimate non-specific reactions, samples were tested in the same way on uncoated plates or on plates coated with uninfected cells.

Conjugation of antibodies with horseradish peroxidase or biotin

Antibodies were conjugated with horseradish peroxidase (HRP) as described by Avrameas and Ternynck (1971). Free peroxidase was separated by gel chromatography with Sephacryl S-300 (Pharmacia, Freiburg), non-coupled globulins by affinity chromatography on Con A–Sepharose (Lannèr et al., 1978). Antibodies were biotinylated as outlined by Peters et al. (1985) with Biotin-X-NHS dissolved in dimethylformamide (Sigma, Munich).

Competition ELISA

Serial dilutions of MAbs (ascitic fluids) were first reacted with optimal working dilutions of conjugated competitive MAbs. The mixtures were then transferred to ELISA plates coated with semipurified BEC and incubated for 60 min at 37°C.

Biotinylated MAbs were detected using avidin coupled with HRP (Sigma, Munich).

SDS-gel electrophoresis and immunoblotting

Proteins of purified BEC (10 μg per slot) were separated in vertical 10% polyacrylamide gels (Laemmli, 1970). The protein bands were made visible by silver staining (Merill et al., 1981) or transferred to nitrocellulose membranes by Western blotting (Towbin et al., 1979) and detected with HRP-conjugated anti-IgG antisera and HRP color developing reagent (Biorad, Munich).

BEC antigen detection by ELISA

To overcome non-specific reactions and low sensitivity, multiple test concepts were investigated. Nevertheless, the procedures were in principle the same. For antigen capture, polyclonal and monoclonal antibodies were used at optimal working dilutions and coated directly onto microplates. Buffers, washings and indicator (TMB) were identical. To compare the specificity and sensitivity of different approaches, the following samples were titrated on coated plates: BEC-infected BEL cell culture fluids (HA titer 1:256), fecal samples scored positive or negative by HI and EM, and additionally bovine rotavirus-infected cell culture fluids. Antigen detection was accomplished by conjugated antibodies or uncoupled antibodies, and the relevant anti-species IgG antisera coupled with HRP.

RESULTS

Characterization of polyclonal and monoclonal antibodies

Neutralizing antibody titers in inactivated sera from guinea pigs and rabbits were 1:5120 and 1:40 000, respectively; HI titers 1:512 and 1:4000, respectively. Non-specific reactions with 2–6% of the specific titer were seen in ELISA, but kaolin treatment and Protein A affinity chromatography reduced non-specific binding significantly, without loss of specific activity.

Titers of antibodies extracted from yolk were 1:256 by HI and 1:25 600 in ELISA. Non-specific reactions in ELISA amounted to 16% of the specific binding. Neither ion exchange chromatography with DEAE–Sephacel nor gel-filtration on Sephacryl S 300 was suitable to reduce background reactions.

Five out of 908 tested hybridoma supernatants were positive by ELISA. Only two colonies, however, proved to be BEC specific, one of them additionally with neutralizing activity. No HI-positive colony was detected. Positive hybridomas were cloned by limiting dilution and propagated in vitro or in the peritoneal cavity of BALB/c mice. The immunoglobulin subclass of all clones was IgG_1.

TABLE 1

Reciprocal titers of polyclonal and monoclonal antibodies in neutralization tests (NT), hemagglutination inhibition (HI) and ELISA on plates coated with semipurified bovine enteric coronavirus (BEC), fetal bovine lung cells (BEL) or on uncoated plates. Immunoblotting experiments were done with purified BEC

Antibody	Treatment	NT	HI	ELISA			Immunoblotting
				BEC	BEL	Uncoated plate	
Rabbit immune serum	Inact.	40 000	40 000	512 000	10 240	5 120	+
	+kaolin	n.d.	n.d.	819 000	320	320	
	+Prot. A	n.d.	n.d.	819 000	n.d.	4 096	
Guinea pig immune serum	Inact.	5 120	512	128 000	8 000	1 000	+
Yolk antibodies	Isopropanol	n.d.	256	25 600	n.d.	4 096	n.d.
MAK 1/1	sf.	1 024	neg.	20 480	neg.	neg.	neg.
	Ascites	8.0×10^6	neg.	6.5×10^7	5 120	640	neg.
	Prot. A	n.d.	neg	1.3×10^7	n.d.	100	neg.
MAK 2/1	sf.	neg.	neg.	2 560	4	neg.	neg.
	Ascites	neg.	neg.	1.0×10^6	320	320	neg.
	Prot. A	neg.	neg.	409 000	n.d.	40	neg.

sf. = serum-free cell culture supernatant; inact. = heat inactivation (56°C, 30 min.); Prot. A = Protein A eluates; n.d. = not done.

The neutralizing MAb (1/1) bound in ELISA with titers 32–64 times higher than the non-neutralizing MAb. By competition ELISA, the neutralizing subclones hindered the non-neutralizing ELISA-positive MAb (2/1) from binding to BEC, whereas MAb 2/1 could not inhibit binding of neutralizing MAb.

In immunoblotting experiments, polyclonal sera bound to viral proteins with molecular weights of 120, 100, 57 and 26 kDa. Both MAbs failed to react. Table 1 summarizes the results of all antibodies tested.

Establishment of an ELISA for antigen detection

Attempts to develop an assay based only on polyclonal antisera failed because of low sensitivity and specificity. Therefore, some variations were tested to combine monoclonal and polyclonal antibodies. The modifications used are listed in Table 2 and the abbreviations of the test combinations (roman/arabic numbers) refer to this table. The MAbs proved to be best suited for antigen

TABLE 2

Antibody combinations used for the establishment of BEC antigen ELISA. Uncoupled antisera were detected by the relevant HRP-conjugated antispecies globulins at a dilution of 1:1000. Biotinylated antibodies were detected by peroxidase-conjugated avidin (diluted 1:1000). In all combinations tested, TMB was used as indicator

Coating	Detection system	
	Detection antibody 1 (Anti-BEC)	Detection antibody 2
I MAb BEC 1/1 ascites 1:5000	1 Yolk antibodies 1:10 000	Anti-species globulins (HRP-conjugated) 1:1000
II MAb BEC 1/2 ascites 1:5000	2 Guinea pig I.-serum 1:1000	or
III MAb BEC 1/1 + MAb BEC 1/2 ascites 1:5000	3 Rabbit immune serum (kaolin treated) 1:2000	Avidin (HRP-conjugated) 1:1000
	4 Rabbit immune serum (Prot. A purif.) 1:10 000	
	5 Rabbit I.-serum-HRP 1:2000	
	6 Rabbit I.-serum-Biot. 1:400	
	7 MAb BEC 1/1-Biot. 1:1000	
	8 MAb BEC 1/2-Biot. 1:500	

capture. Setting up an optimal detection system, the use of koalin-treated rabbit antiserum in the indirect assay (III/3) resulted in the highest extinction for BEC, whereas background reactions were not observed at all. Purification of the antiserum on Protein A (III/4) did not improve the signal. When the same rabbit serum was conjugated with HRP or biotin (III/5 and III/6) the extinctions decreased more rapidly. The use of yolk antibodies (III/1) or guinea pig serum (III/2) gave unacceptable background values.

According to these results, the indirect test combination III/3 was chosen with a monoclonal capture system and a polyclonal detection system. It proved

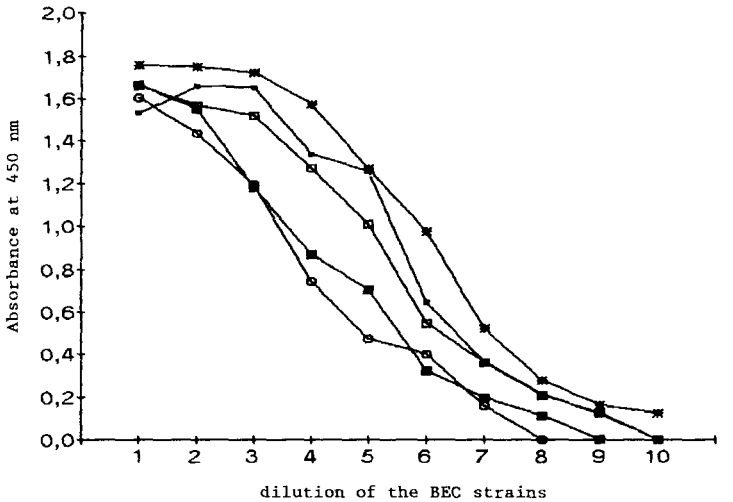

Fig. 1. Log (2) titration curves of the following cell culture-adapted BEC strains in the optimal ELISA configuration III/3. The starting dilution was 1:5. HA titers of the strains are in parentheses. —∗—, BEC V270/83 Munich (HA: 1:256); —•—, BEC 800 Holland (HA: 1:128); —□—, BEC P 1239 France (HA: 1:64); —○—, BEC V410/83 Munich (HA: 1:32); —■—, BEC RIT-Genoval Belgium (HA: 1:32).

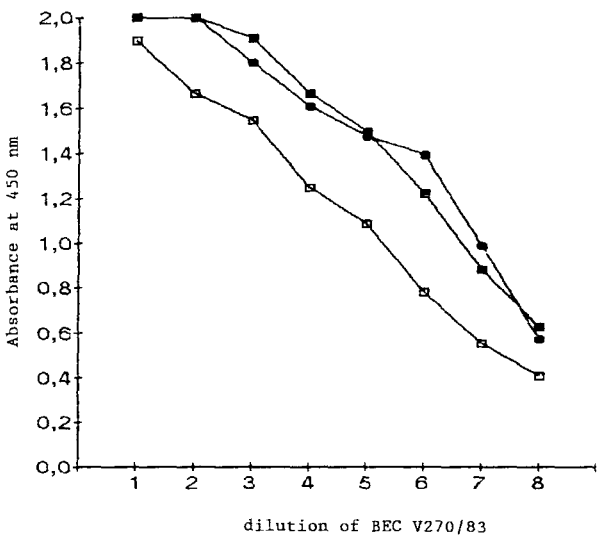

Fig. 2. Log (2) titration curves of the cell culture-adapted BEC V270/83 Munich in the monoclonal/polyclonal combined ELISA configurations. —•—, I/3; —□—, II/3; —■—, III/3. The starting dilution was 1:5.

to be superior for antigen detection to all other tested variations. The specificity of this assay was confirmed by blocking the polyclonal rabbit detection system with the BEC-specific yolk, guinea pig and MAbs. Negative fecal samples, rotavirus or other bovine viruses failed to react in this assay.

Titration curves of cell culture-adapted BEC strains were of similar shape, but the extinction values depended on their HA titers (Fig. 1). For the detection of BEC V270/83, it did not matter whether one or two MAbs were used for antigen capture (Fig. 2). MAb 1/1 (I/3) was slightly more effective than MAb 2/1 (II/3), but an amplifying effect of the combination of both MAbs (III/3) was not seen. This could generally be confirmed when fecal samples were tested. However, in a small number of specimens there were striking differences in the reactivity of the two antibodies with the BEC isolates. The catching efficacy of the two MAbs was significantly different. The weak reaction of one MAb was compensated for by the other. With two samples, the neutralizing MAb 1/1 failed to react entirely. In other samples, MAb 2/1 bound

TABLE 3

Extinction values of some fecal samples obtained with different catching antibody combinations as shown in Table 2. Samples were tested at dilutions of 1:5-1:40

Dilution	No. 486			No. 571			No. 354		
	I/3	II/3	III/3	I/3	II/3	III/3	I/3	II/3	III/3
1:5	2.000	2.000	2.000	1.658	1.672	1.673	0.930	0.837	0.828
1:10	2.000	2.000	2.000	1.493	1.386	1.608	0.491	0.405	0.417
1:20	2.000	2.000	2.000	1.077	0.980	1.205	0.152	0.191	0.234
1:40	2.000	1.966	2.000	0.762	0.704	0.924	0.076	0.128	0.162
Dilution	No. 526			No. 390			No. 451		
	I/3	II/3	III/3	I/3	II/3	III/3	I/3	II/3	III/3
1:5	2.000	1.753	2.000	1.050	0.348	0.880	0.943	1.862	1.849
1:10	2.000	1.492	2.000	0.629	0.184	0.549	0.839	1.688	1.496
1:20	1.934	1.107	2.000	0.323	0.103	0.301	0.743	1.309	1.068
1:40	1.912	1.045	1.892	0.170	0.066	0.194	0.560	0.783	0.715
Dilution	No. 392			No. 436			No. 341		
	I/3	II/3	III/3	I/3	II/3	III/3	I/3	II/3	III/3
1:5	0.028	1.352	1.005	0.000	1.378	0.733	0.358	1.838	1.754
1:10	0.016	0.882	0.551	0.000	0.905	0.458	0.206	1.627	1.351
1:20	0.006	0.472	0.223	0.000	0.437	0.211	0.116	1.059	0.909
1:40	0.000	0.276	0.175	0.000	0.264	0.130	0.096	0.597	0.382

more weakly, but the signal was always clearly detectable. Some representative results are summarized in Table 3. The samples are grouped according to their reaction with the two MAbs.

DISCUSSION

ELISA techniques have been developed for the detection of antigens of many different viruses (Yolken, 1982). Most of these assays use polyclonal antibodies only, whereas MAbs have been introduced mainly to increase specificity. However, in the case of BEC, only two ELISAs using polyclonal antibodies exclusively have been described to date (Ellens et al., 1978; Reynolds et al., 1984). Like others (Crouch et al., 1984), we also failed to establish such an assay for antigen detection. This failure was due to high background reactions and low sensitivity. Crouch et al. (1984) explain their failure with antibodies to rotaviruses that may have been elicited during the immunization period or with antibodies directed against cellular components as being a result of the use of insufficiently purified immunogens. In this report, we used highly purified antigen preparations which were not reactive with MAbs directed against cellular components (C.-P. Czerny, unpublished data). Antibodies to rotaviruses in the sera we used being a reason for non-specific reactivity are unlikely as the antisera did not react with rotaviruses to a greater extent than with other viruses (bovine herpesvirus type I or vaccinia virus) or with uninfected cell cultures. Even with uncoated plates, a similar behaviour was seen. This non-specific binding is frequently observed at low dilutions, but can be overcome by higher dilutions of the antisera. This procedure, however, was not effective in establishing an ELISA for the detection of BEC antigens as with higher dilutions of polyclonal catching antibodies sensitivity decreased sharply. Purification of these catching antibodies on Protein A–Sepharose or treatment with kaolin did not improve the results. One approach to overcome these problems is purification of the antibodies by affinity chromatography on purified BEC virions, as described by Sato et al., (1984). We preferred as an alternative the production of highly specific MAbs. Out of 908 tested hybridoma colonies, only five were positive by ELISA. After thorough testing, only two of them proved to be BEC specific whereas the others cross-reacted with cellular components or were reactive with uncoated plastic plates ("sticky antibodies"). The BEC-specific MAbs were used as catching antibodies and could be coated directly onto the plates. For antigen detection, rabbit immune serum proved to be superior in terms of sensitivity as optimal specificity was guaranteed by the catching MAbs. However, untreated serum could not be used. Kaolin treatment turned out to be optimal compared to other procedures. Antiserum produced in guinea pigs or antibodies extracted from yolk gave unacceptably high background reactions that could not be reduced by different techniques. The use of conjugated (HRP or biotin) rabbit serum resulted in decreased sensi-

tivity, indicating that the anti-species IgG conjugate serves as an additional amplifier.

Vautherot and Laporte (1983) reported different reaction patterns of MAbs with BEC field strains. Our studies confirm these results. Therefore, both MAbs were essential for antigen capture. They did not complement one another additively, but compensated for each other when one antibody was less effective in antigen capture due to epitope differences between BEC strains. The protein specificity of the two MAbs could not be determined because they failed to react in immunoblotting. The epitopes recognized by the MAbs are obviously conformation dependent and are probably located close together on the virion. This was seen in competition ELISA, where the neutralizing MAb hindered the non-neutralizing MAb from binding. Hogue et al. (1984) as well as Deregt et al. (1987) have shown that BEC proteins of 120 and 100 kDa are responsible for neutralization. Some of the isolates tested probably lost the epitope recognized by the neutralizing MAb 1/1.

The above-described ELISA for BEC detection in feces has some advantages over other techniques. The assay is simple and can be performed rapidly, blocking is not necessary, and the results can be read with the naked eye or evaluated by measuring optical densities. This objectivity is important compared to electron microscopy and HA/HI. The results of comparative investigations will be presented in a subsequent paper.

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