Vol. 99 No. 2 February 2005

Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology

ENDODONTOLOGY

Editor: Larz S. W. Spångberg

Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after "one-visit" endodontic treatment

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Objective. To assess the in vivo intracanal microbial status of apical root canal system of mesial roots of human mandibular first molars with primary apical periodontitis immediately after one-visit endodontic treatment. The residual intracanal infection was confirmed by correlative light and transmission electron microscopy.

Study design. Sixteen diseased mesial roots of mandibular first molars were treated endodontically, each in one visit. Mesio-buccal canals were instrumented using stainless steel hand files and mesio-lingual canals with a nickel-titanium rotary system. The canals were irrigated with 5.25% sodium hypochlorite (NaOCl) during the instrumentation procedures, rinsed with 10 mL of 17% ethylenediamine tetraacetic acid (EDTA), and obturated with gutta-percha and zinc oxide eugenol cement. Thereafter, the apical portion of the root of each tooth was removed by flap-surgery. The specimens were fixed, decalcified, subdivided in horizontal plane, embedded in plastic, processed, and evaluated by correlative light and transmission electron microscopy.

Results. Fourteen of the 16 endodontically treated teeth revealed residual intracanal infection after instrumentation, antimicrobial irrigation, and obturation. The microbes were located in inaccessible recesses and diverticula of instrumented main canals, the intercanal isthmus, and accessory canals, mostly as biofilms.

Conclusions. The results show (1) the anatomical complexity of the root canal system of mandibular first molar roots and (2) the organization of the flora as biofilms in inaccessible areas of the canal system that cannot be removed by contemporary instruments and irrigation alone in one-visit treatment. These findings demonstrate the importance of stringent application of all nonantibiotic chemo-mechanical measures to treat teeth with infected and necrotic root canals so as to disrupt the biofilms and reduce the intraradicular microbial load to the lowest possible level so as to expect a highly favorable long-term prognosis of the root canal treatment.

(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2005;99:231-52)

Apical periodontitis is a disease of persistent microbial infection of the root canal system of the affected tooth. The microbial etiology of the disease has long been well established.[1,2] The infected and necrotic root canal system acts as a selective habitat for the causative organisms[3] that grow mostly in sessile biofilms, aggregates, and coaggregates in which they are embedded in an extracellular matrix material.[4,5] Therefore, the logical goal of treatment of the disease has been to eliminate or substantially reduce the microbial population within the root canal system and to prevent reinfection by a tight seal of the root canal space. The disruption of the biofilms and reduction of the microbial load are achieved by a combination of mechanical instrumentation, irrigation with various tissue lytic and microbicidal solutions, and application of antimicrobial medicaments in the root canal.

Traditionally, the mechanical cleaning, enlarging, and shaping of the root canal have been done using a stainless steel hand instrument involving a filing and reaming motion. Since the introduction of instruments made out of highly flexible alloys to endodontics,[6] nickel-titanium (NiTi) rotary systems[7-10] have achieved widespread popularity among clinicians because of their convenience

The authors are indebted to Mrs Margrit Amstad-Jossi for skillful and efficient technical assistance.
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Received for publication Aug 23, 2004; returned for revision Sep 27, 2004; accepted for publication Oct 7, 2004.
1079-2104/$ - see front matter

doi:10.1016/j.tripleo.2004.10.005

and superiority in negotiating curved root canals as compared to stainless steel instruments. The NiTi instrumentation has been evaluated and found to be effective in maintaining the original shape of the canal.[11] It is assumed that NiTi instruments shape the apical portion of root canals so as to facilitate a better flow of the irrigants into the area compared to instrumentation with traditional stainless steel files. It has also been suggested[12,13] that the size of apical instrumentation may be important for the effective removal of microorganisms from the canal. Molar canals have been found to be cleaner "...by means of counting the remaining surface debris..." when instrumentation reached a size #45 apical file.[14] On the other hand, when the mesial canals communicate with each other (about 40% of the cases), it was difficult to obtain culture-negative canals even after having enlarged the apical portion of the canal to a size #60 apical file.[15]

Teeth without apical radiolucency at the time of root canal treatment have a higher rate of success than those with apical radiolucent lesions.[16] Further, teeth that are root-filled after obtaining a negative microbial culture have a better long-term prognosis than those root-filled with microbes known to be present in the root canals at the time of obturation.[17,18] In any case, for maximum long-term clinical success of root canal treatment, it is desirable to obturate the canal without any infectious agent present or with a microbial load reduced to a level that cannot be detected by contemporary growing techniques. However, in the absence of routine microbial monitoring of root canals before obturation, clinicians can expect an optimal outcome of treatment only when the treatment procedures follow a protocol that had established efficient microbial reduction of the infected canals.

The efficiency of reducing the quantity of intracanal microorganisms has been investigated during various stages of root canal treatment by techniques using traditional stainless steel hand files[13,19-23] and modern rotary NiTi instruments.[15,24,25] The microbiological method of evaluation in these studies consisted of taking intracanal samples with paper-points at various stages of treatment, culturing the samples, and quantitatively estimating the viable microbes in the samples. These studies indicate that negative microbial cultures can be expected with high repeatability only after thorough instrumentation and rinsing of the root canals with sodium hypochlorite (NaOCl) and ethylenediamine tetraacetic acid (EDTA), and application of an interappointment microbicidal intracanal dressing such as calcium hydroxide ($Ca(OH)_2$). The therapeutic action of the microbicidal dressing requires sufficient time to be effective and thus cannot be completed in 1 session or "one-visit endodontic treatment."

The complexity of the root canal system formed by the main canals, an isthmus communicating between them, accessory canals, apical ramifications, and anastomoses[26,27] cannot be adequately visualized by radiographs[28,29] or resolved by microcomputed tomography.[30,31] Therefore, the problem of microorganisms surviving in the inaccessible, remote areas of the canal system that are beyond the reaches of contemporary sampling procedures remains a serious concern. Thus, the purpose of this study was to assess the in vivo intracanal microbial status of the apical root canal system of mesial roots of human mandibular first molars with primary apical periodontitis immediately after a one-visit endodontic treatment by 2 different instrumentation techniques, namely, a NiTi rotary instrumentation and a standard step-back procedure using stainless steel hand K-files. The presence of residual intracanal microorganisms was confirmed by modern correlative light and transmission electron microscopy (TEM).

MATERIAL AND METHODS

Subjects and specimens

Patients considered for inclusion in this study had teeth with pulpal necrosis and apical periodontitis. The criteria for tooth selection included presence of a distinct periapical radiolucent lesion, a negative response to thermal pulp sensitivity test, presence of enough coronal tissues for adequate isolation of the tooth with rubber dam, and no prior endodontic treatment on the involved tooth. All the subjects were treated in accordance with the Helsinki Declaration (www.cirp.org/library/ethics/helsinki). Informed consent of each patient was obtained after explaining the clinical procedures and risks involved and clarifying all questions raised by the patients. Only mature mandibular first molars were used in this study. A total of 16 mesial roots of mandibular first molars were treated endodontically, each in one visit, and apical surgery was performed immediately thereafter (Table I). Only 1 tooth per patient was sampled.

Clinical procedures

All clinical procedures were done by 2 experienced endodontists. After local anesthesia and isolation of the tooth by rubber dam, disinfection of the tooth and the rubber dam was done using 5.25% of NaOCl with a circular movement starting on the tooth and going outwards to the rubber dam. Thereafter, carious tissues were removed and endodontic access cavities were prepared with sterile high-speed carbide burs. Gates Glidden drills were used for coronal flaring with copious irrigation using 5.25% NaOCl. Thereafter, the working lengths of the root canals were determined using 2 stainless steel hand-files (size 15) and the Root Zx Electronic Apex Locator (J. Morita & Co, Tustin, Calif).

Table I. Summary of the clinical data of subjects whose teeth were investigated

No.	*Specimens*	*Patient*	*Age*	*Sex*	*Tooth*	*Group*
01	MX-01	IM	40	M	19 (36)	Trial
02	MX-02	MES	18	F	19 (36)	Trial
03	MX-03	AL	21	F	30 (46)	Trial
04	MX-04	MM	42	F	19 (36)	Trial
05	MX-05	JCC	20	M	19 (36)	Trial
06	MX-06	MIS	48	F	30 (46)	Trial
07	MX-07	EG	42	M	30 (46)	Trial
08	MX-08	YM	42	F	30 (46)	Trial
09	Mx-09	JSL	25	F	19 (36)	Trial
10	MX-10	MIH	27	F	30 (46)	Trial
11	MX-11	VC	21	M	30 (46)	Trial
12	MX-12	MR	33	F	19 (36)	Trial
13	MX-13	CS	39	M	19 (36)	Trial
14	MX-14	MT	30	F	30 (46)	Trial
15	MX-15	DSP	13	F	19 (36)	Trial
16	MX-16	HA	27	F	30 (46)	Trial
17	MX-17	RMA	33	M	30 (46)	+ Control
18	MX-18	AQR	32	M	19 (36)	+ Control
19	MX-19	NAC	53	F	30 (46)	+ Control
20	MX-20	JVP	67	M	19 (36)	+ Control
21	MX-21	JTG	14	F	21 (34)	−Control
22	MX-22	MRA	15	F	28 (44)	−Control
23	MX-23	MRA	15	F	21 (34)	−Control

Mesio-angled and/or disto-angled radiographs were taken to visualize and confirm the presence of 2 separate canals. The mesial canals were divided into 2 groups. All mesio-buccal canals were instrumented using a standardized technique with K hand files (Kerr Manufacturing Co, Romulus, Mich) until the apical preparation reached a #25 apical size. Thereafter, a step-back technique was used by withdrawing 1 mm of successive file tips from that of the previous until a #40-sized hand file was reached (3 mm from the working length). All mesio-lingual canals were instrumented with a standardized technique using Lightspeed (Lightspeed Technology Inc, San Antonio, Tex) NiTi instruments until a #40 apical size was reached. Copious irrigation with 5.25% NaOCl during the instrumentation procedure and a final rinse with 10 mL of 17% EDTA were done in both groups. All canals were subsequently obturated by lateral condensation of gutta-percha and zinc-oxide eugenol cement (Roth 801). Thereafter, the access cavities were sealed with Cavit (3M ESPE, AG Seefeld, Germany) and posttreatment control radiographs were taken.

On completion of the root canal treatment on each patient, a full-thickness intrasulcular flap was raised under strict antiseptic conditions so as to expose the periapical lesion and the surrounding tissues. An osteotomy was performed until the apical third of the mesial root could be clearly identified. Using a # ¼ round sterile carbide bur, a shallow groove was prepared on the buccal surface of the exposed apical root portion. The groove was meant to identify the buccal aspect of the resected root-segment so as to recognize and orientate the mesio-buccal canal that had been hand-file prepared. The apical third of the mesial root was carefully removed using a Endo-Zekria (Maillefer, Ballaigues, Switzerland) high-speed sterile carbide bur operating with saline irrigation. The flap was repositioned and sutured. The patients were given oral and written postsurgical instructions. The entire clinical procedures were done in one visit.

Controls

The apical third of the distal root of 4 mandibular first molars with necrotic pulps and radiographic signs of apical periodontitis and scheduled for extraction were used as positive controls (Table I). The apical third of 3 clinically healthy mandibular first bicuspid roots served as the negative controls (Table I). The teeth had to be removed for orthodontic reasons. The clinical status of the 3 teeth was determined by a combination of physical examination, radiography, and tooth sensitivity test. Physical and radiographic examinations revealed that the teeth were caries-free and asymptomatic. Pulp sensitivity was determined by the application of a cotton pellet with EndoIce (Hygenic Corporation, Akron, Ohio) to the cervical area of the involved tooth. All 3

Table II. Summary of certain histo-antomical features of the apical root canal system of the specimens presented in Table I.

Anatomical feature	*n*	*%*
Total number of specimens	16	100
Number of specimens with mesio-lingual canal	16	100
Number of specimens with mesio-buccal canal	16	100
Number of specimens with isthmus	11	69
Number of specimens with accessory canal(s)	8	50

teeth were positive for cold thermal sensitivity test. A caries-free, asymptomatic, and cold-sensitive tooth was assumed to be clinically healthy with a vital pulp.

Tissue processing

Immediately after removal, the surgically removed apical third was fixed by immersion in a modified Karnovsky's fixative, consisting of 2% paraformaldehyde and 2.5% glutaraldehyde buffered in 0.02 M sodium cacodylate for several days.[32] Thereafter, the specimens were decalcified in solutions containing 0.25 mol/L EDTA and 4% glutaraldehyde. The demineralized specimens were orientated with the help of the "buccal groove" prepared during surgery and were subdivided into about 0.3-mm-thick to 0.5-mm-thick horizontal discs. As the buccal groove was not extending to the full length of the root tip, an insignificant portion of each root disc on the buccal aspect was cut off with a sharp razor blade for clear orientation during further processing of the specimen. The apical root discs were post-fixed in 1.33% osmium tetroxide (OsO_4), in s-collidine, dehydrated in ascending grades of ethanol and embedded in Epon® (Fluka AG, Buchs, Switzerland). The correlative light and TEM investigation was performed as described in detail before.[33] Briefly, from each Epon block, 10 to 12 survey sections of about 1 μm to 2 μm thickness and from selected blocks serial sections were prepared using glass or histodiamond knives (Diatome AG, Biel, Switzerland) and the Reichert Ultracut E microtome (Leica, Glattbrugg, Switzerland). The sections were stained in periodic acid-Schiff (PAS) and methylene blue-Azur II and photomicrographed in a Dialux 20 photomicroscope (Leica) equipped with the digital camera Progress C14 (Jenoptik, Eching, Germany) and a digital imaging system (ImageAccess, Imagic, Glattbrugg, Switzerland). The sections were studied thoroughly in a light microscope for the presence of microorganisms in the root canals and/or sites that are likely to contain microbes such as the presence of neutrophils. Thereafter, such areas in the Epon-blocks were determined for ultrasectioning. The selected tissue sites were target-trimmed and thin-sectioned with the Reichert Ultracut E microtome (Leica). The thin sections were double contrasted with lead and uranium salts[34,35] and examined in a Philips EM400T transmission electron microscope (Philips, Endoven, The Netherlands). The negative micrographs were scanned in a UMAX Powerlook 3000 (UMAX Systems, Willich, Germany) scanner and the digitized electron micrographs were further processed using an image-processing program (Photoshop 7.1; Adobe, San Jose, Calif).

RESULTS

Endodontically treated (trial) specimens

Histologically all the 16 endodontically treated teeth revealed the presence of mesio-lingual and mesio-buccal canals (Table II). Four of them showed confluence towards the apices. Eleven of the 16 specimens revealed communications (isthmus) between the 2 main canals. The root discs of 8 of the 16 specimens showed cross-sectional profiles of varying sizes and numbers of accessory canals and/or anastamosing segments of the root canal system.

Microbial status

In general, microorganisms were found in the complex, apical root canal system of 14 (88%) of the 16 root canal treated specimens (Tables III and IV).

Intracanal infection. Although 14 (88%) of the 16 specimens showed the presence of microbes in the apical root canal system, the organisms were not uniformly distributed and located in all specimens. Eight of the 16 specimens revealed microbes in both the mesio-lingual (hand filed) and mesio-buccal canals (NiTi prepared). Seven of the 8 microbe-positive mesio-lingual and mesio-buccal canals belonged to identical root-specimens. Of the 11 root tips that revealed an isthmus between the 2 main canals, 10 showed the presence of microorganisms within. Accessory root canals were present in 8 of the 16 specimens, of which 5 contained a mixed microbial flora.

As a representative case, one of the specimens in which microorganisms were observed in the main and accessory canals is illustrated radiographically (Fig 1), histologically (Fig 2), and transmission electron microscopically (Fig 3). The specimen originated from the mesial root of the left mandibular first molar of a 40-year-old male patient (MX-1, Table I). The coronally divergent mesio-lingual and the mesio-buccal canals converged toward the apical-most portion of the root (Fig 1, *B*; Fig 2, *A* and *B*). At the section plane of the illustration (Fig 2), signs of canal instrumentation and presence of gutta-percha and sealer components were clearly recognizable. The canals also revealed inaccessible pulp recesses and irregular diverticula that contained a slurry of microbes, organic materials, and dentinal chips. The striking feature of the specimen was

Table III. Microbial status and location of microbial presence (+) or lack of observation (−) assessed by light and transmission electron microscopy in the apical root canal system of the trial specimens shown in table I.

No.	*Specimen*	*MLC*	*MBC*	*IST*	*AC*	*Overall**	*Remarks*
01	MX-01	+	+	N	+	+	Canals confluent
02	MX-02	+	+	+	+	+	
03	MX-03	+	+	+	+	+	
04	MX-04	−	−	−	−	−	
05	MX-05	−	−	+	N	+	Found in TEM only
06	MX-06	−	−	+	N	+	Found in TEM only
07	MX-07	+	−	N	+	+	
08	MX-08	−	−	+	N	+	
09	MX-09	+	+	+	+	+	
10	MX-10	−	+	+	N	+	
11	MX-11	−	−	+	+	+	Canals confluent
12	MX-12	+	+	+	N	+	
13	MX-13	−	−	+	N	+	
14	MX-14	+	+	N	N	+	Canals confluent
15	MX-15	+	+	N	−	+	Canals confluent
16	MX-16	−	−	N	N	−	

MLC, mesio-lingual canal; *MBC;* mesio-buccal canal; *AC,* accessory canal; *IST,* isthmus; *N,* absence of the structural entity.
*Denotes the presence of microbes (+) or lack of observation of such (−) in 1 or more of the 4 anatomical locations examined.

Table IV. Summary of the microbial status of the specimens detailed in Table III.

Microbial status	*n*	*%*
Total number of specimens	16	100
Overall specimens with microbes in the apical canal system	14	88
Number of specimens with microbes in mesio-lingual canal	8	50
Number of specimens with microbes in mesio-buccal canal	8	50
Number of specimens with microbes in isthmus	10	63
Number of specimens with microbes in accessory canals	5	31

the presence of cross-sectional profiles of numerous accessory canals and anastomosing segments of a complex root canal system that were uninstrumented and clogged with microorganisms (Fig 2, *E* and *F*). In TEM (Fig 3) the flora consisted of a mixed microbial population of cocci, rods, and predominantly filamentous organisms. Numerous dividing forms of cocci and rods could be recognized. The flora appeared to be held together by an electron lucent extracellular matrixlike substance. The microbes and the extracellular material clogged the entire lumen of the accessory canal. In random ultrathin sections, the dentinal wall in isolated segments revealed multilayered microbial condensation that resembled biofilms attached to the firm dentinal base. Spirochetes were present in the root canal system of this and another (MX-7, Table II) endodontically treated specimen.

Intra-isthmus infection. In 11 of the 16 specimens, the main canals were found to communicate through an isthmus of varying width. The dentinal wall of the isthmus often showed arcading profiles of Howship's lacunae that were colonized and filled with microorganisms (MX-11, Fig 4). The microbe-clogged, tortuous isthmus often contained "islands" of fibrodentinal structures (Fig 5). Depending on the location and plane of sectioning, the isthmus appeared in segments and sizes that were difficult to be observed in low magnification bright field microscopy (MX-8, Fig 6). However, in TEM, even a very narrow isthmus revealed numerous bacteria and neutrophils, the latter in varying stages of disintegration (Fig 7).

Plant cell−associated infection. One of the specimens (MX-15, Table I), illustrated radigraphically (Fig 8), histologically (Fig 9), and electron microscopically (Fig 10), contained intraradicular foreign particles of vegetable nature (Fig 9) that revealed large plant cells with distinct electron lucent cell walls and disintegrated cellular contents (Fig 10). The intracanal and intra-isthmus bacteria in this case were not only found in the root canal space but were also present within the disintegrated plant cells (Fig 10). Whereas some plant cells were free of bacteria, others showed varying numbers of coccoid bacterial cells of which many were in dividing forms (Fig 10, *B*).

Positive controls

All the 4 nontreated, necrotic root canals that served as positive controls revealed the presence of a mixed

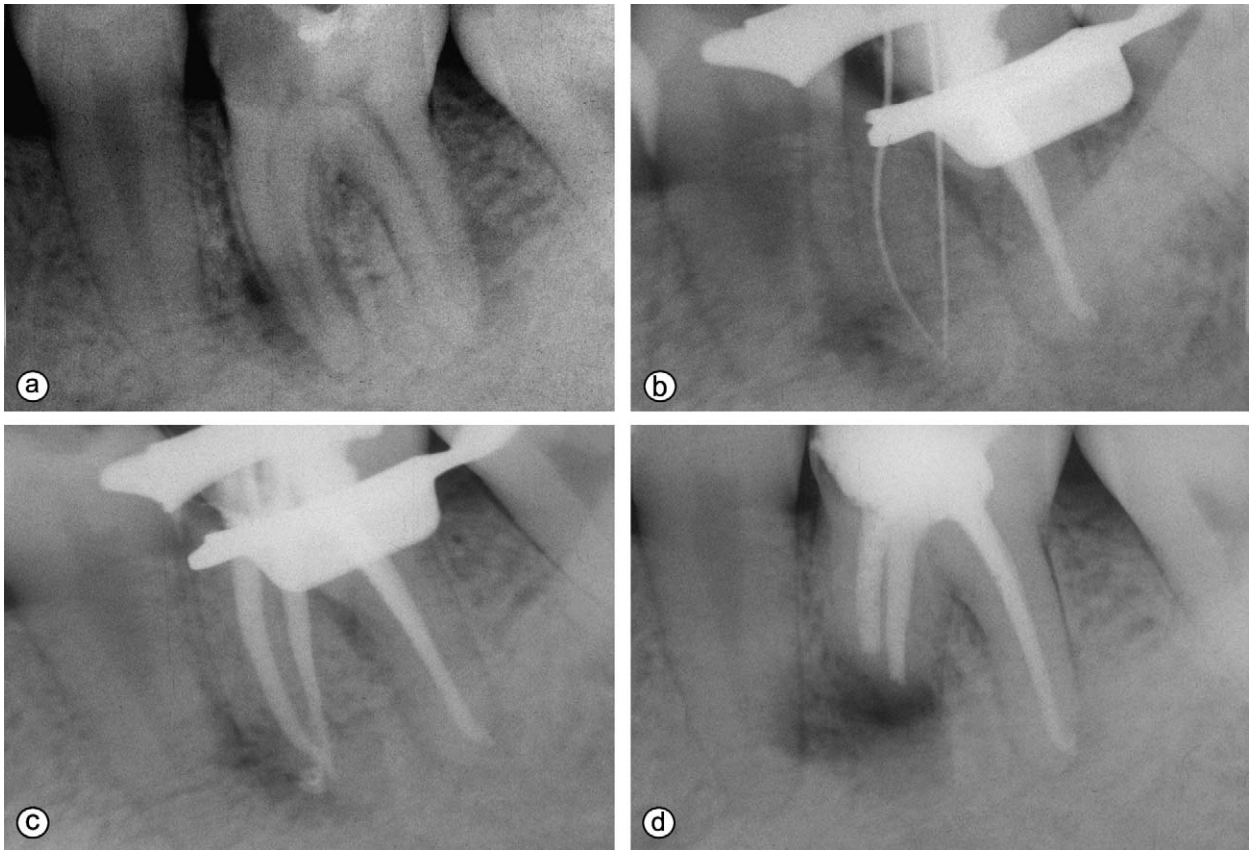

Fig 1. Radiographs of a left mandibular left first molar (MX-01, Table I) with primary apical periodontitis (**A**). The mesio-angled images recorded during instrumentation (**B**) and after obturation (**C**) reveal 2 major root canals of the mesial root. The apical third of the root was removed by surgery immediately after the root canal treatment in "one visit" (**D**). The histological and transmission electron microscopic findings of the apical root segment are illustrated in Fig 2 and Fig 3, respectively.

microbial flora both in the main and accessory canals. Histologically, the main canals of the 4 specimens contained disintegrated radicular pulp tissue and a loosely distributed mixed microbial flora. The accessory canals, on the other hand, were clogged with microorganisms. One of the specimens (MX-19, Table I) is illustrated in detail (Figs 11 to 14). The loose and clogged distribution of the intraradicular contents of the main and accessory canals respectively, could be observed even in low magnifications (Fig 11). In high magnifications the flora consisted of cocci, rods, and filamentous organisms (Fig 12). An exceptional feature of the specimen MX-19 was the presence of ovoid and filamentous microbial cells that were several times larger in size than that of the bacterial cells. Even in a light microscope (Fig 12), these cells appeared to exist throughout the canal lumen, but they seemed to be attached to the entire pulpal dentinal wall so as to form multileveled sessile biofilm (Fig 13). In the midcanal region these cells appeared loosely mixed with the much smaller sized bacterial cells. In higher magnification (Figs 13 and 14), they appeared as dividing forms of ovoid and elongated microbial cells that were often held together to form chains. These organisms were found to be about 10 to 12 μm in diameter and possessed distinct electron lucent cell walls and electron dense nuclear area. These structures had the characteristic morphology of fungus.

Negative controls

No microorganisms were encountered in the canals of the 3 root tips that originated from clinically healthy teeth. These showed typical healthy radicular pulp that consisted of peripheral cuboidal odontoblasts, soft connective tissue, and neurovascular bundles. In morphological works, absence of something cannot be convincingly illustrated with validity, so no illustration of negative controls of healthy radicular pulp is presented.

DISCUSSION

Because microorganisms are the essential etiological agents of primary apical periodontitis[1,2] and also the major cause of posttreatment apical periodontitis,[33,36-41] the cherished goal of treatment of the disease has been

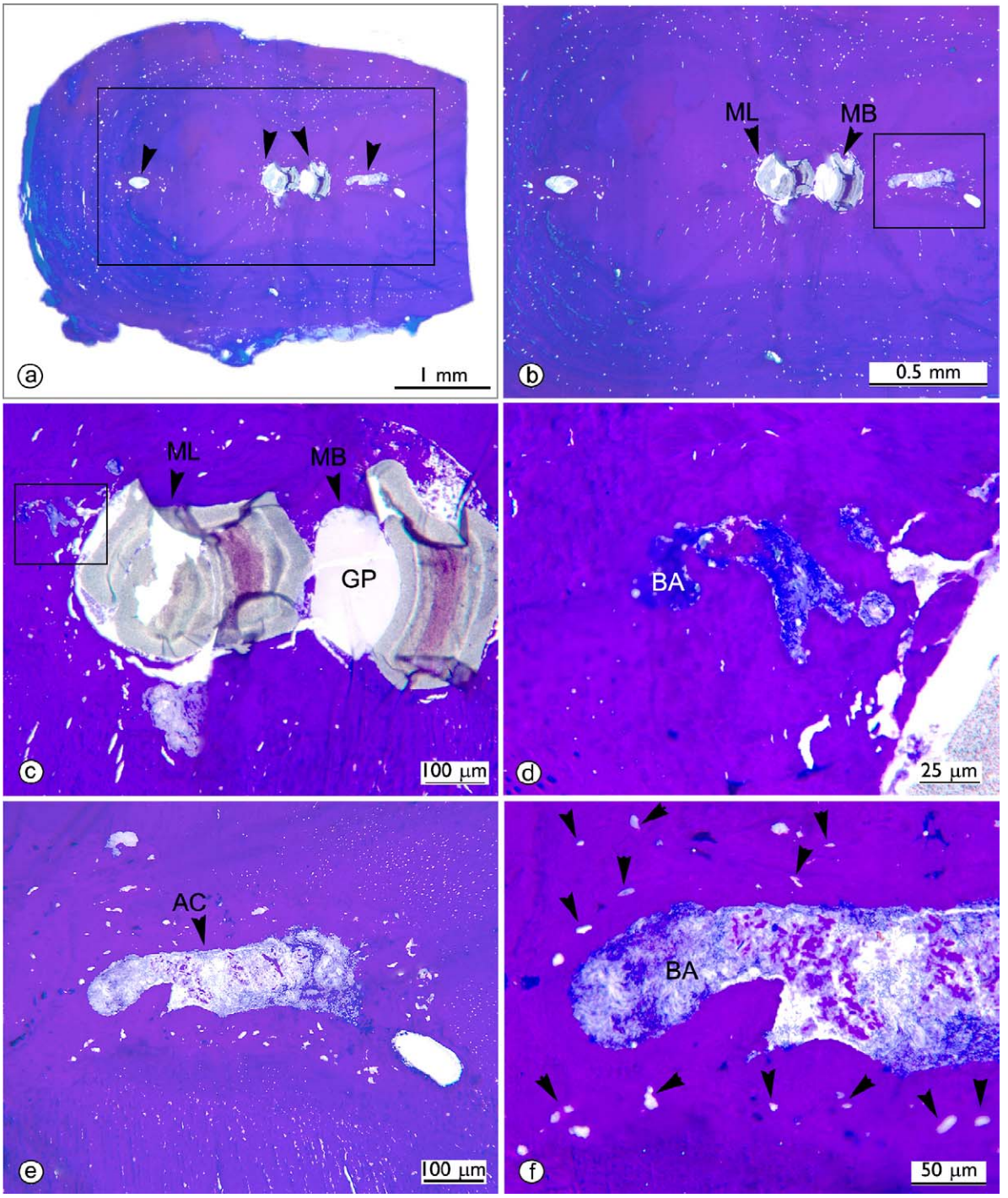

Fig 2. Photomicrograph of a transverse section (**A**) through the apical portion of the mesial root of the tooth removed by surgery from the radiolucent area in Fig 1, **D**. The rectangular demarcated are in **A** is magnified in **B**. The mesio-lingual (ML) and mesio-buccal (MB) canals (magnified in **C**) communicate and are root-filled (GP). The rectangular demarcated area in **B** is magnified in **E**. The main canals show recesses and diverticulations; those in the rectangular demarcated area in **C** are magnified in **D**. One noninstrumented accessory canal (AC in **E**) is enlarged in **F**. Note the diverticulation of ML in **D** and the larger accessory canal (**E**, **F**) clogged with bacteria (BA); the transmission electron microscopic view of the latter is shown in Fig 3. Black arrowheads in **F** show cross-sectioned profiles of anastomoses of the root canal system. Original magnifications: **A**, ×16; **B**, ×40; **C** and **E**, ×100; **D**, ×400; **F**, ×260.

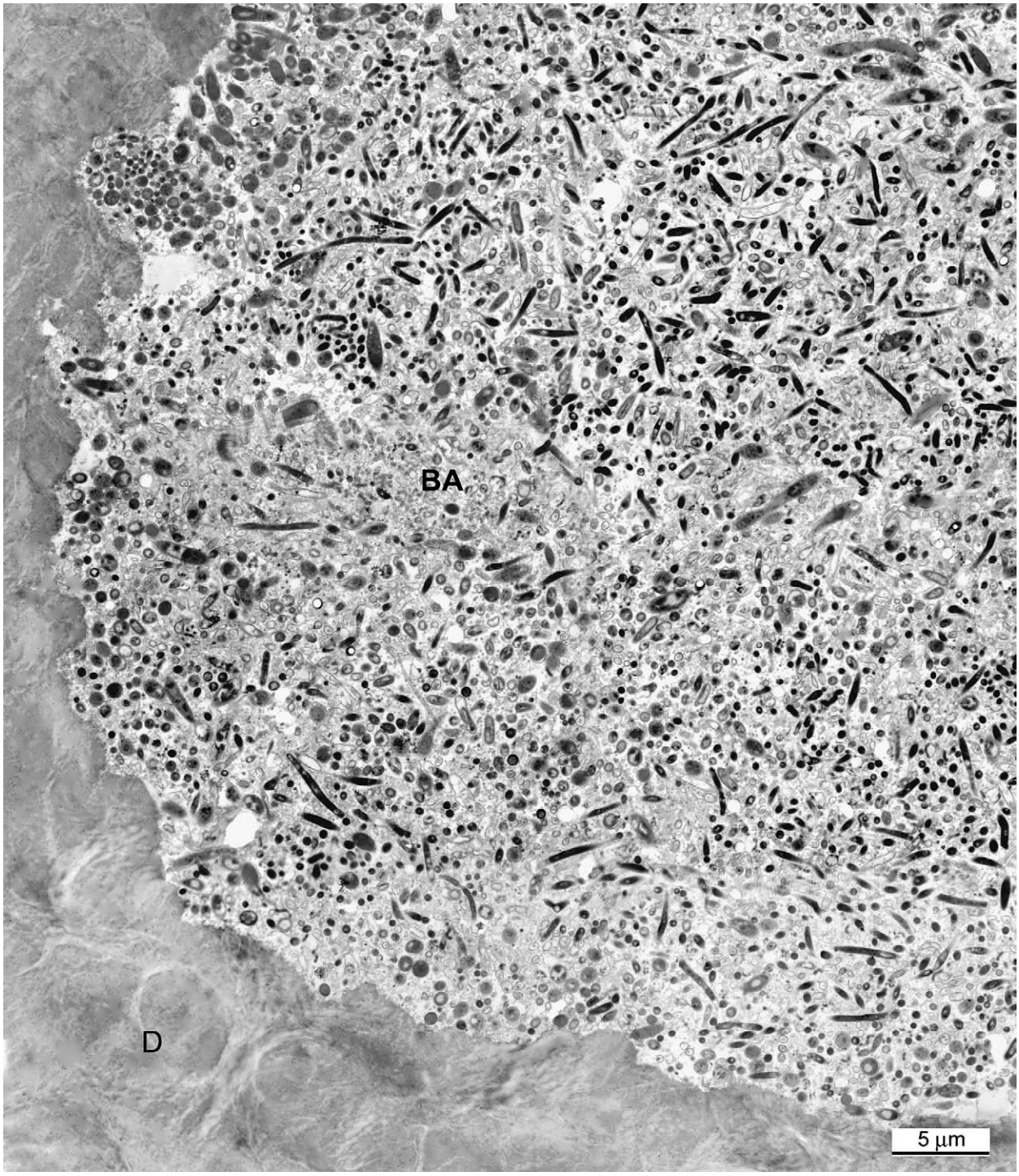

Fig 3. The composite transmission electron micrograph shows bacterial mass in the noninstrumented necrotic accessory canal of Fig 2, **E** and **F**. Note the filamentous bacteria (BA) sectioned in various planes and embedded in an extracellular matrix material. D = Dentine. Original magnification ×3200.

a total elimination of the intraradicular infection and prevention of reinfection. Whether this ideal goal can be achieved or not with contemporary instruments and procedures, particularly in a one-visit treatment schedule, is an ongoing topic of debate.[42,43] This study provides morphological evidence that 14 of the 16 (88%) mandibular molars that were root-treated in a single session harbored intracanal microorganisms

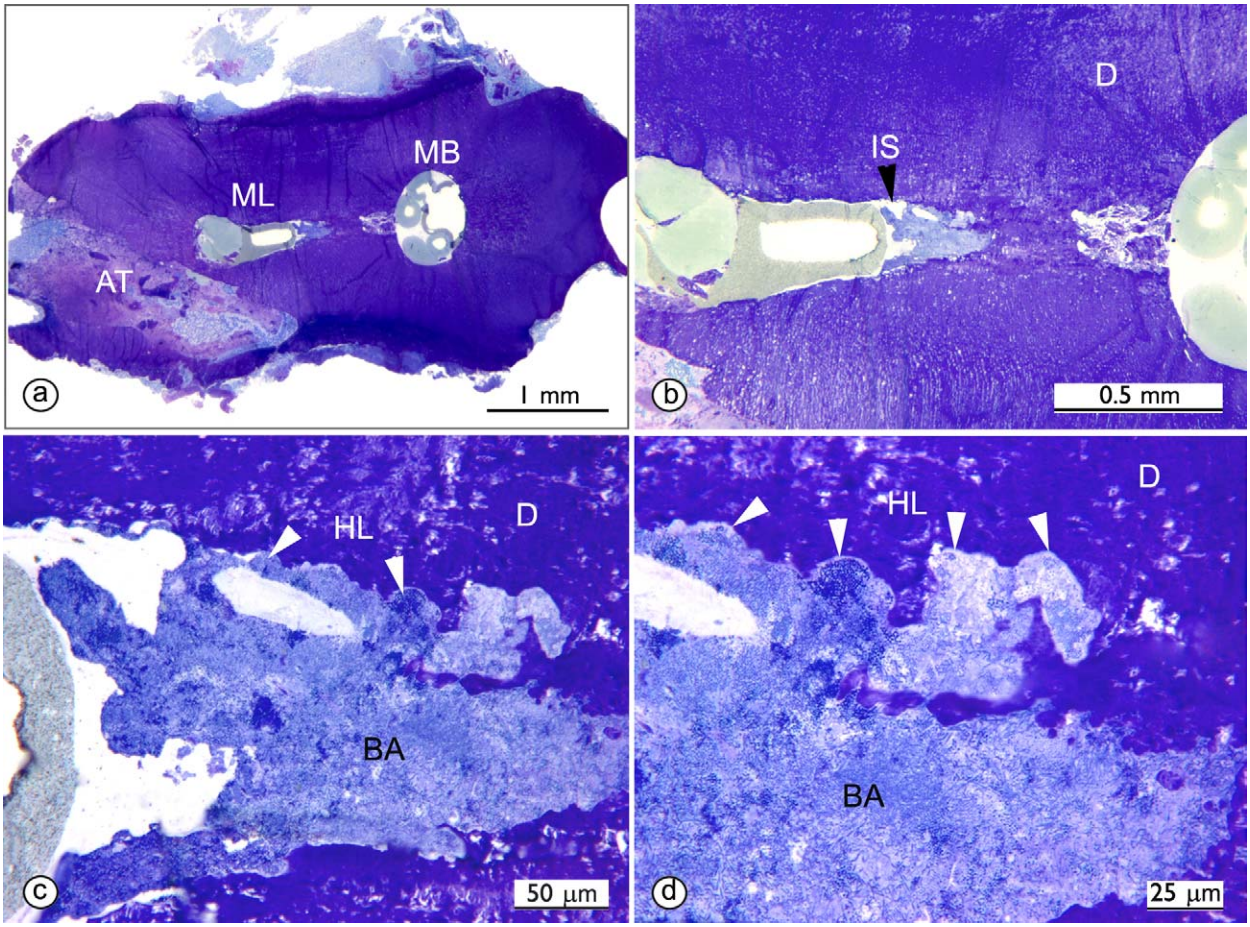

Fig 4. Light microscopic view of a transverse section through the apical portion of the mesial root of a right mandibular first molar (MX-11, Table I). The surgical artifact (AT) into the root dentine did not reach or damage the instrumented mesio-lingual and mesio-buccal canals, which were incompletely obturated with gutta-percha cones. The isthmus (IS) connecting the canals is magnified in **B;** the area indicated with the black arrowhead is further magnified in stages in **C** and **D**, respectively. Note the uninstrumented isthmus with arcading profiles of Howship's lacune (HL) clogged with blue stained bacterial mass (BA). A transmission electron microscopic view is shown in Fig 5. Original magnifications: **A**, ×16; **B**, ×44; **C**, ×240; **D**, ×400.

immediately after completion of the treatment. Further, it reconfirms the long-known anatomical complexity of the apical root canal system[26,27] that probably is the most important factor that makes the task of total elimination of intracanal microbes difficult and allows for the presence of residual infection posttreatment.

This study assessed the intracanal microbial status of endodontically treated teeth immediately after such treatment in one visit for which 2 commonly used instrumentation techniques were used on separate canals of first human mandibular mesial roots. It should be emphasized that the study did not attempt to make a comparison between the 2 instrumentation techniques. Nevertheless, it is worth noting that the outcome of treatment, in terms of the microbial status, was almost identical with both techniques. This finding is completely in agreement with the results of a study[24] that compared the efficiency of the 2 techniques in reducing intracanal microbes.

The fact that 14 of the 16 root-treated teeth contained residual intracanal infection after instrumentation, irrigation with NaOCl followed by EDTA, and obturation demonstrates the difficulty of total eradication of microbes from infected canals. There were 2 specimens in which intracanal microbes were not detected after the treatment. It does not mean that the apical canals of the 2 specimens were free of microbes. It is very much likely that the 2 cases also contained residual microbes that were not encountered by the methods used. In the 14 specimens that were positive for microbes, the organisms were located not only in the inaccessible recesses and diverticula of instrumented main canals but also in the isthmus and accessory canals that could not be cleaned by the treatment procedures. The presence of

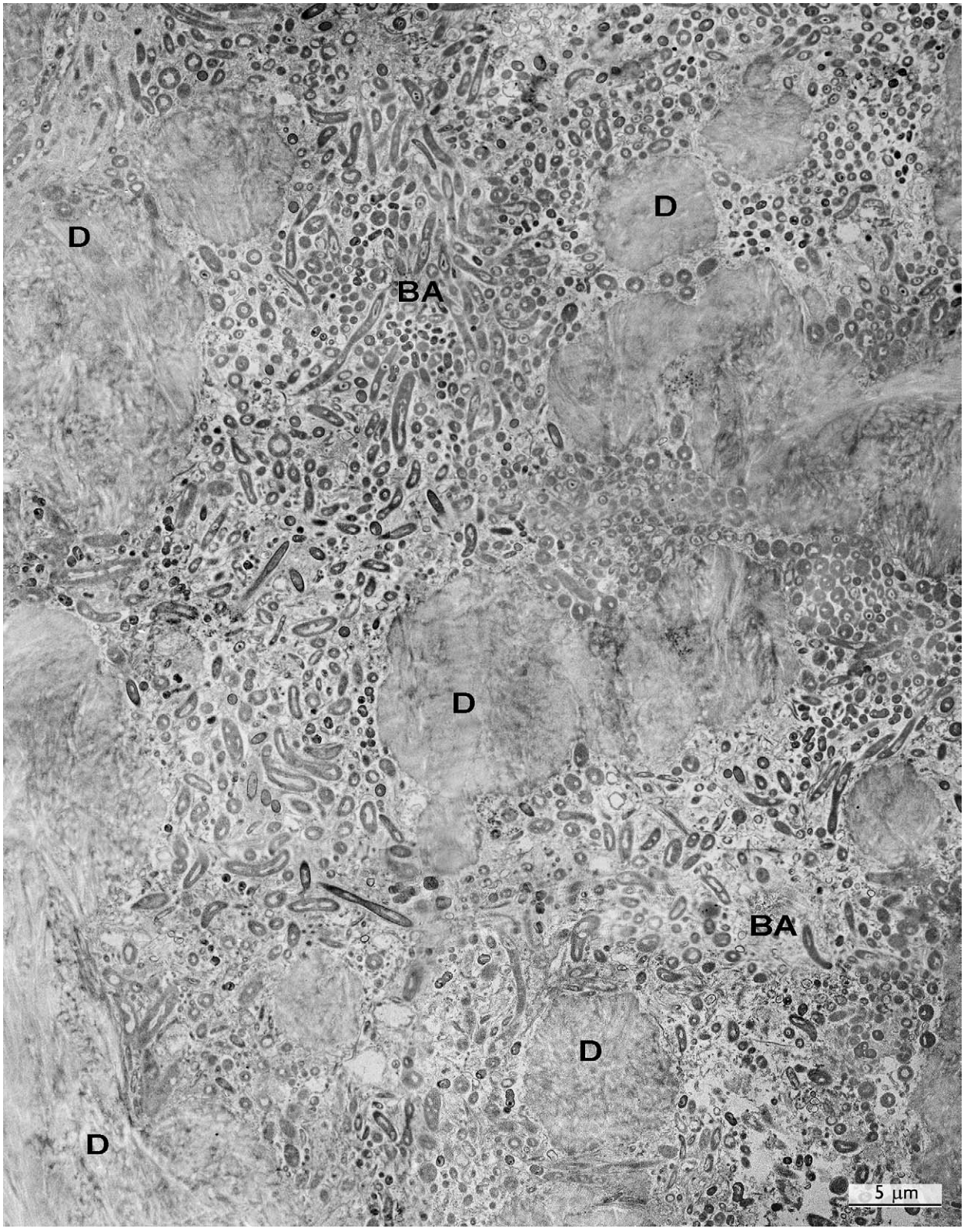

Fig 5. Transmission electron micrograph of bacterial (BA) mass in Fig 4, **D**, in the tortuous space of the isthmus that reveals numerous "islands" of fibro-dentinal (D) structures. Original magnification: ×3200.

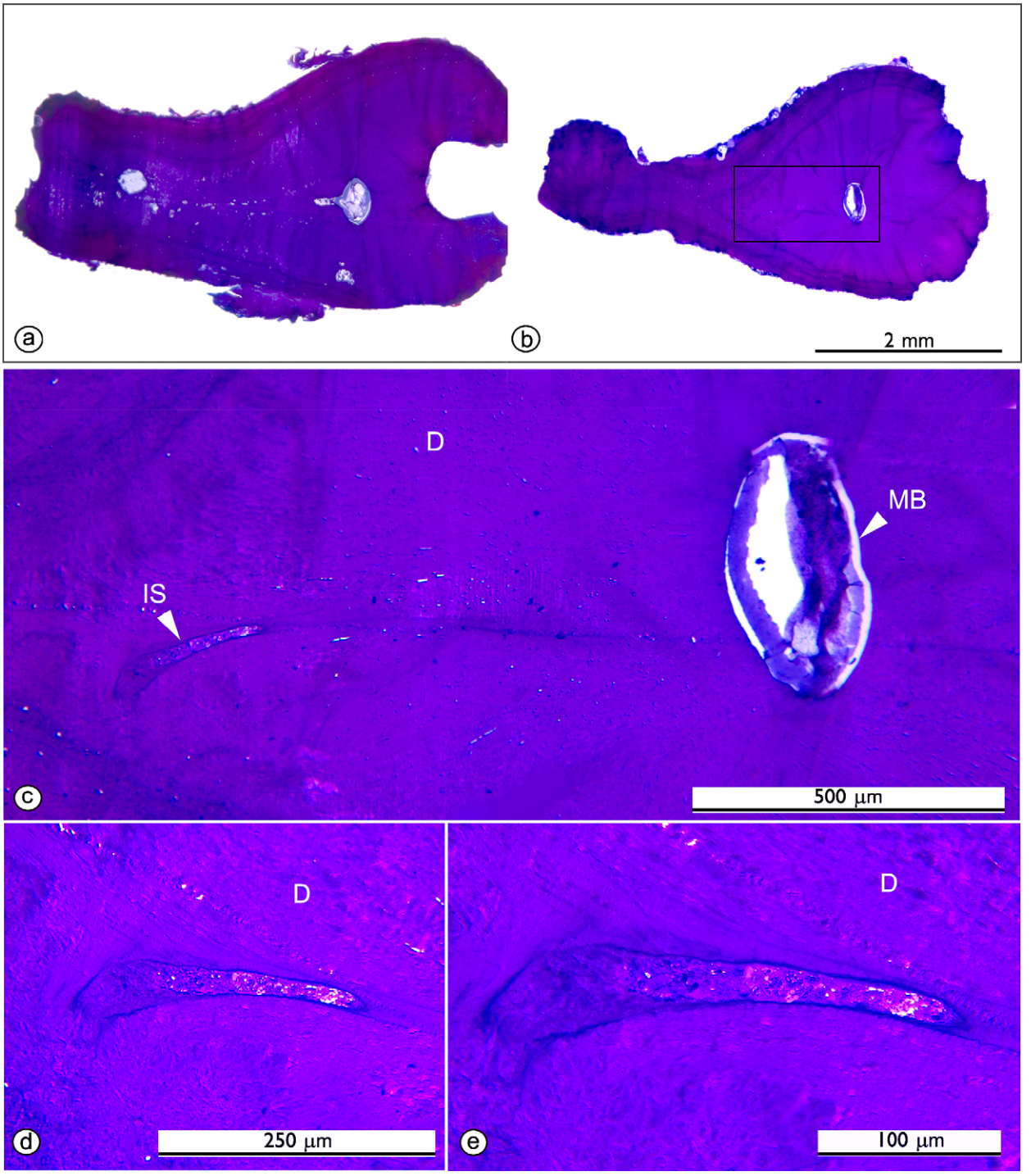

Fig 6. Light photomicrographs of apical root discs of the mesial root of a right mandibular first molar (MX-08, Table I). The identification notch is on the buccal aspect in **A**. The mesio-lingual (ML) and mesio-buccal (MB) canals are wide apart in the more cervical section (**A**) but only the mesio-buccal canal is still present in the more apical root segment (**B**). The rectangular demarcated area in **B** is magnified in **C**. The tangentionally cut segment of a very narrow isthmus (IS) is further magnified in stages in **D** and **E**. The contents of the isthmus cannot be resolved at the respective magnifications but are distinctly clear in the electron micrograph of the area presented in Fig 7. Original magnifications: **A** and **B**, ×15; **C**, ×100; **D**, ×180; **E**, ×300.

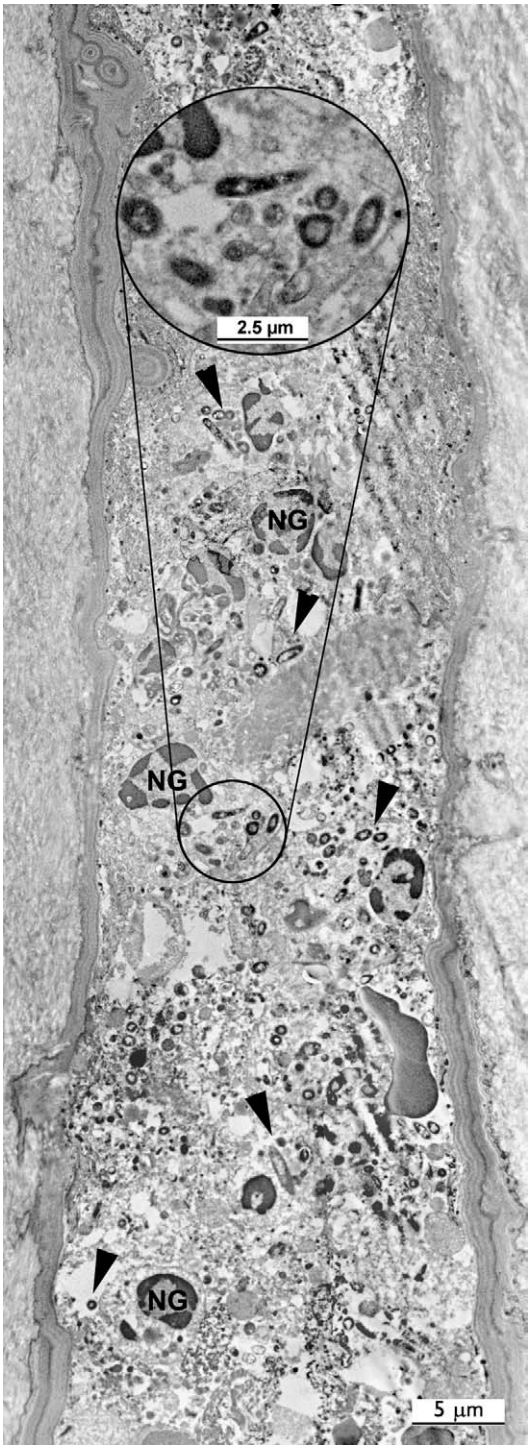

Fig 7. A composite transmission electron micrograph of the isthmus shown in Fig 6, **E**. The narrow uninstrumented isthmus shows plenty of bacteria and numerous neutrophils (NG) in varying stages of disintegration. The small circular demarcated area is magnified in the inset. Note the distinct (arrowheads) filamentous bacterial profiles sectioned in various planes. Original magnification: ×2800; inset ×5600.

mitotic forms of cocci and rods is a clear indication of the viability of the organisms at the time of surgery and fixation of the specimens. The observation of numerous neutrophils, even in the very narrow isthmus of some specimens, indicates migration of the cells from blood vessels of the apical periodontitis into the necrotic and infected root canal system by chemotaxis against intracanal microbial antigens. It is also a sign of presence of a fluid phase of the apical canal system. These observations are fully in accordance with similar findings reported before.[4] For technical reasons, the possibility that microbes remained in dentinal tubules of the treated teeth was not investigated in this study. Morphologically, the trial specimens contained a polymicrobial flora consisting of cocci, rods, filaments, and spirochetes. This is fully in accordance with the findings of investigations using the precise correlative microtechniques on the apical root canal system of teeth with asymptomatic posttreatment apical periodontitis.[33,44]

One of the 14 teeth with posttreatment residual infection also showed the presence of foreign particles of vegetable origin in the canal system. The particles were most probably derived from vegetable foods of nonleguminous nature that got trapped preoperatively in the pulp chamber of the tooth that had an open carious cavity. Cellulose containing food particles and clinical materials (paper-points, cotton wool) is known to be transported through the root canal system into the periapical area to cause granulomatous lesions by a foreign body reaction that is commonly referred to as cellulose granuloma.[45,46] Further, the foreign particles passing through an infected root canal can also carry microorganisms with them, as was the case with the specimen MX-15, into the periapical area and allow a foreign body–related biofilm[47] to grow around them. This has been clearly demonstrated to occur also in the periapex of a human tooth.[45]

The intracanal microorganisms existed, both in the positive controls and in the uninstrumented areas of the main treated canals and untouched accessory canals of the 14 trial specimens, as matrix-embedded multileveled structures attached to the dentinal pulpal walls and also as planktonic cells, aggregates, or coaggregates of the species suspended in the fluid phase of the infected and necrotic root canal. This is consistent with the observations made in 1987 that "electron microscopically a condensed bacterial layer could be identified on the dentinal wall of the root canal which when light microscopically visible, gave the palisade structure of bacterial plaques adhering to tooth surfaces."[4] In a contemporary microbiological sense, this means that the undisturbed intracanal flora of an infected tooth with apical periodontitis is mostly organized as a matrix-embedded collection of multispecies organisms in

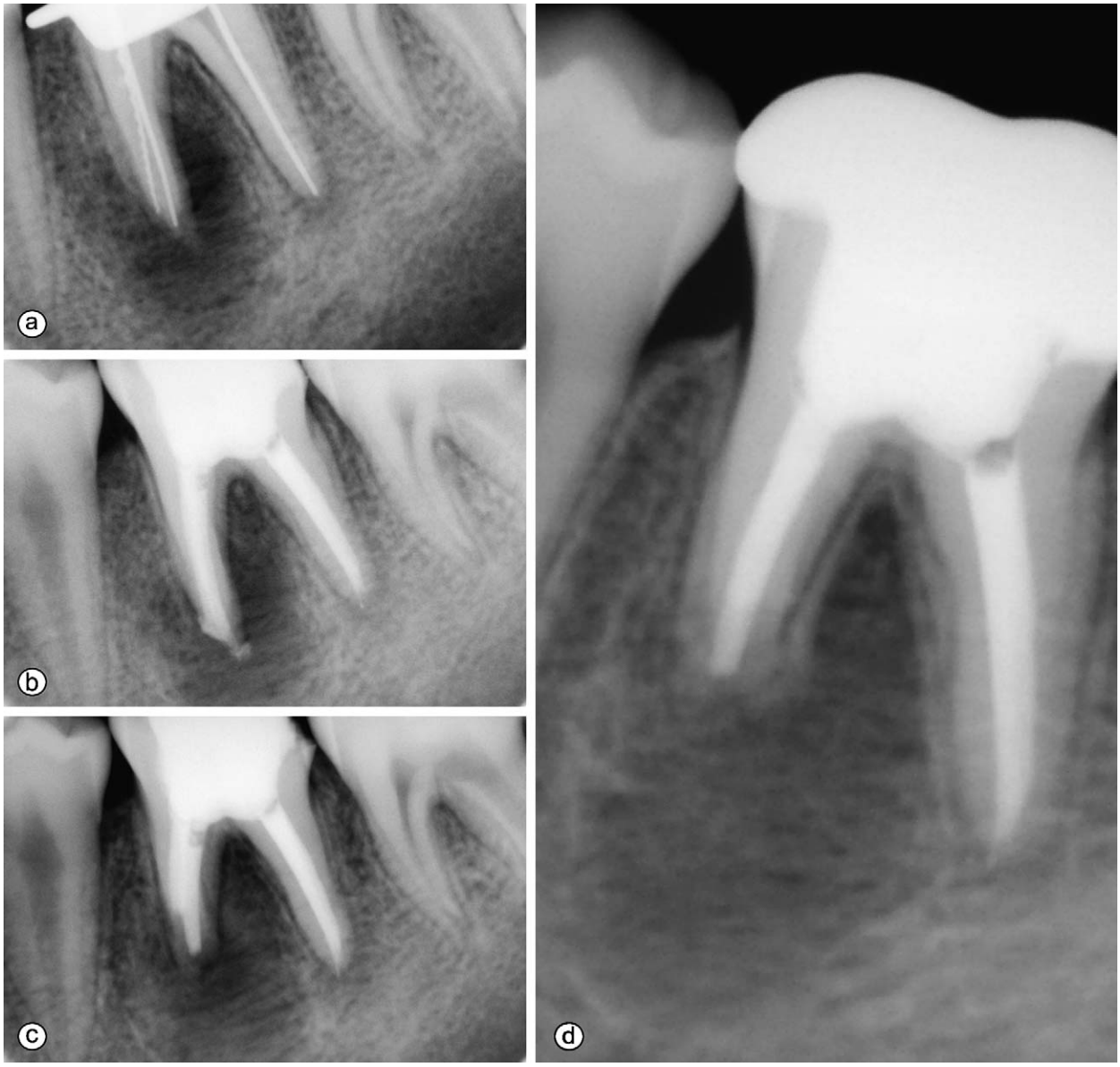

Fig 8. Radiographs (**A** and **B**) of the mandibular left first molar (MX-15, Table I) with primary apical periodontitis during root canal instrumentation (**A**) and postobturation (**B**). Note the slight extrusion of the root-filling material into the periapical area, which was removed by apical surgery (**C**). Eight months after surgery (**D**), there is evidence of bone regeneration. The histological and transmission electron microscopic findings of the apical root segment are illustrated in Fig 9 and Fig 10.

microecosystems that are immobilized on dentinal surface[48] as a biofilm,[5,47] which cannot be eradicated by host defenses or chemotherapy alone. Therefore, an effective reduction of the mostly sessile intracanal flora can be achieved only by mechanical dislocation of the biofilm, cleaning and planing of the infected inner dentine using hand and/or rotary instruments, washing away of the organisms and debris by irrigating with antimicrobial solutions, and deposition of microbicidal dressings in the canals.[19]

Several investigators have studied the effect of the crucial mechanical measures of instrumentation and irrigation with sterile distilled water or physiological saline and achieved substantial quantitative reduction of the intracanal flora.[49-51] Current data on the efficiency of reducing the microbial load from infected canals using stainless steel hand files and associated antimicrobial procedures come from a series of carefully designed and executed studies[19-22,52] that evaluated the effect of individual steps in the treatment procedures on the

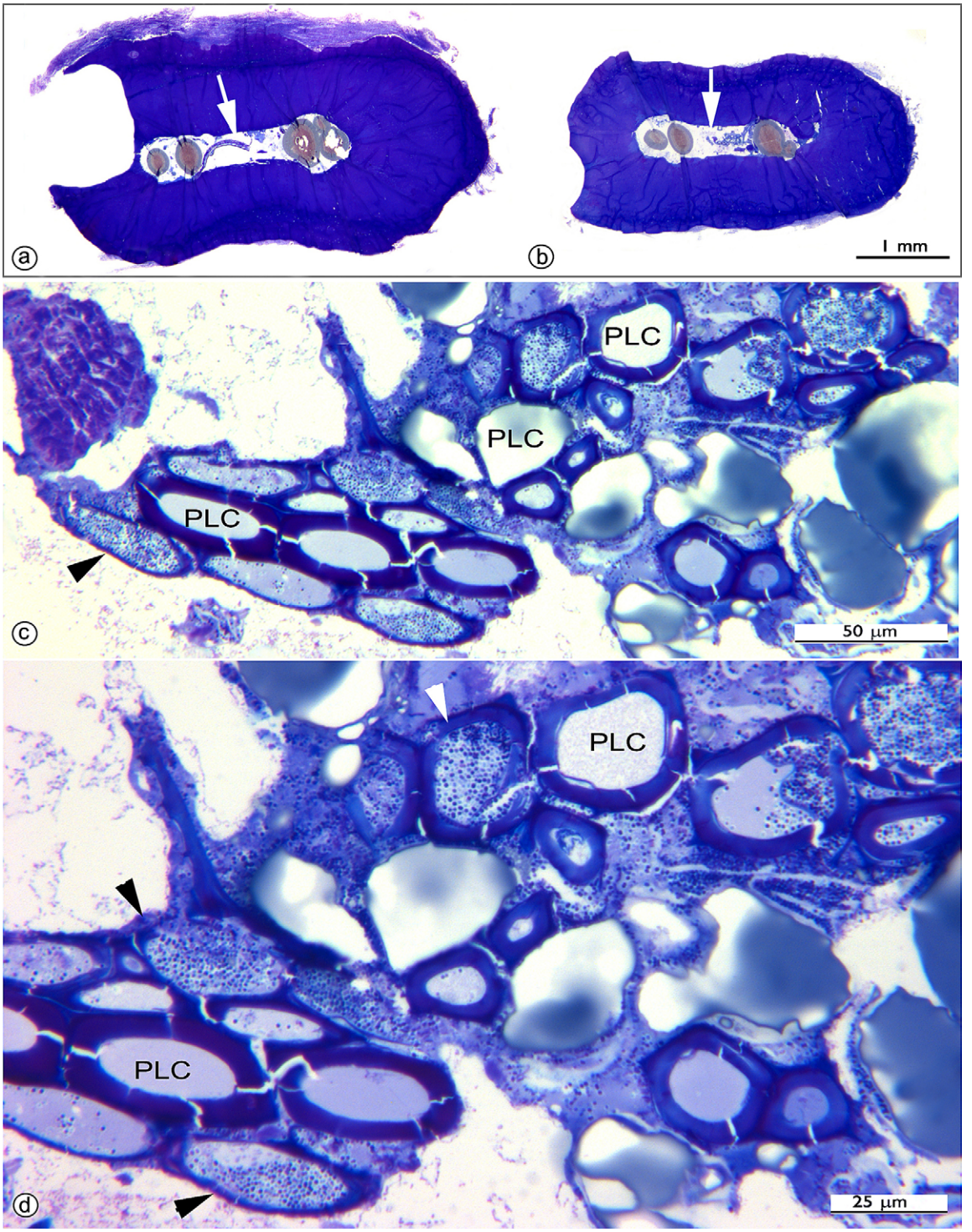

Fig 9. Photomicrographs of 2 transverse sections through the apical portion of the mesial root of the mandibular left first molar (MX-15, Table I) removed by surgery from the radiolucent area in Fig 8, **C**. The identification notch is on the buccal side. Note the circular profiles of gutta-percha cones incompletely obturating the mesio-lingual and mesio-buccal canals that are connected by a wide isthmus. The latter contains blue-stained materials from the arrowed area in **B** that are magnified in stages in **C** and **D**, respectively. Note the remnants of plant cells (PLC) with distinct cell walls and disintegrating cytoplasmic area that in many contain dot-like structures resembling cocci. Original magnifications: **A** and **B**, ×16; **C**, ×520; **D**, ×800.

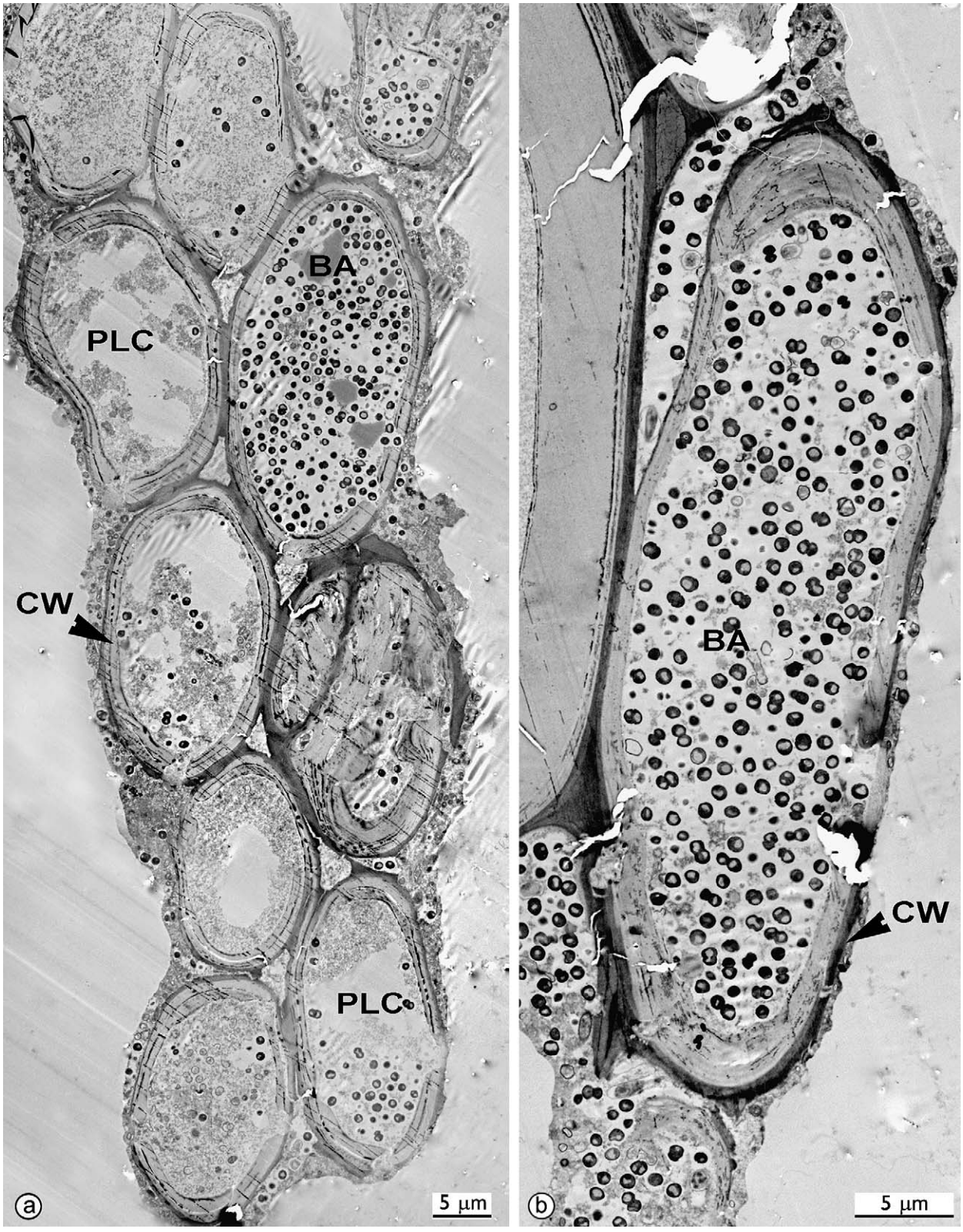

Fig 10. Transmission electron micrographs of the plant cells (PLC) depicted in Fig 9, **D**. Note the distinct electron lucent cell walls (CW). The intracellular area of the PLC reveal varying numbers of coccoid bacteria (BA), many among them show mitotic forms in the higher magnification in **B**. Original magnifications: **A**, ×2000; **B**, ×3400.

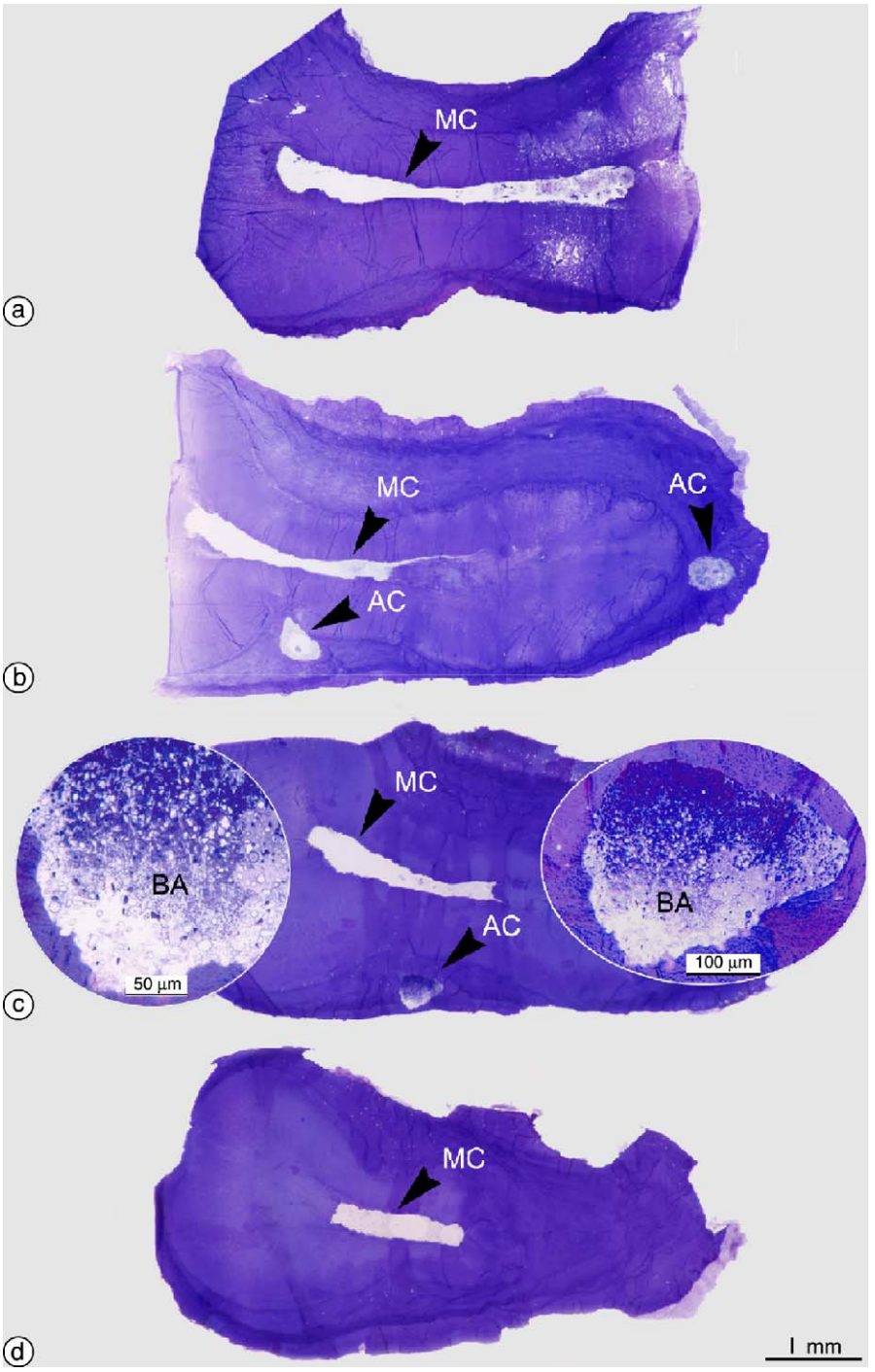

Fig 11. Photomicrographs (**A**-**D**) of sequential transverse sections at varying distances in corono-apical direction from the apical portion of a necrotic, noninstrumented and nonobturated distal root of a mandibular first molar (MX-19, Table I) with primary apical periodontitis (positive control). The main canal (MC) contains microorganisms. Note the accessory canals (AC) in **B** and **C**. The AC on the right-hand side in **B** is magnified in Fig 12. The demarcated AC in **C** is magnified in stages in the right and left insets, respectively, in **C**. Note the AC is clogged with bacteria (BA). Original magnifications: **A**-**D**, ×15; right inset in **C**, ×110; left inset in **C**, ×180.

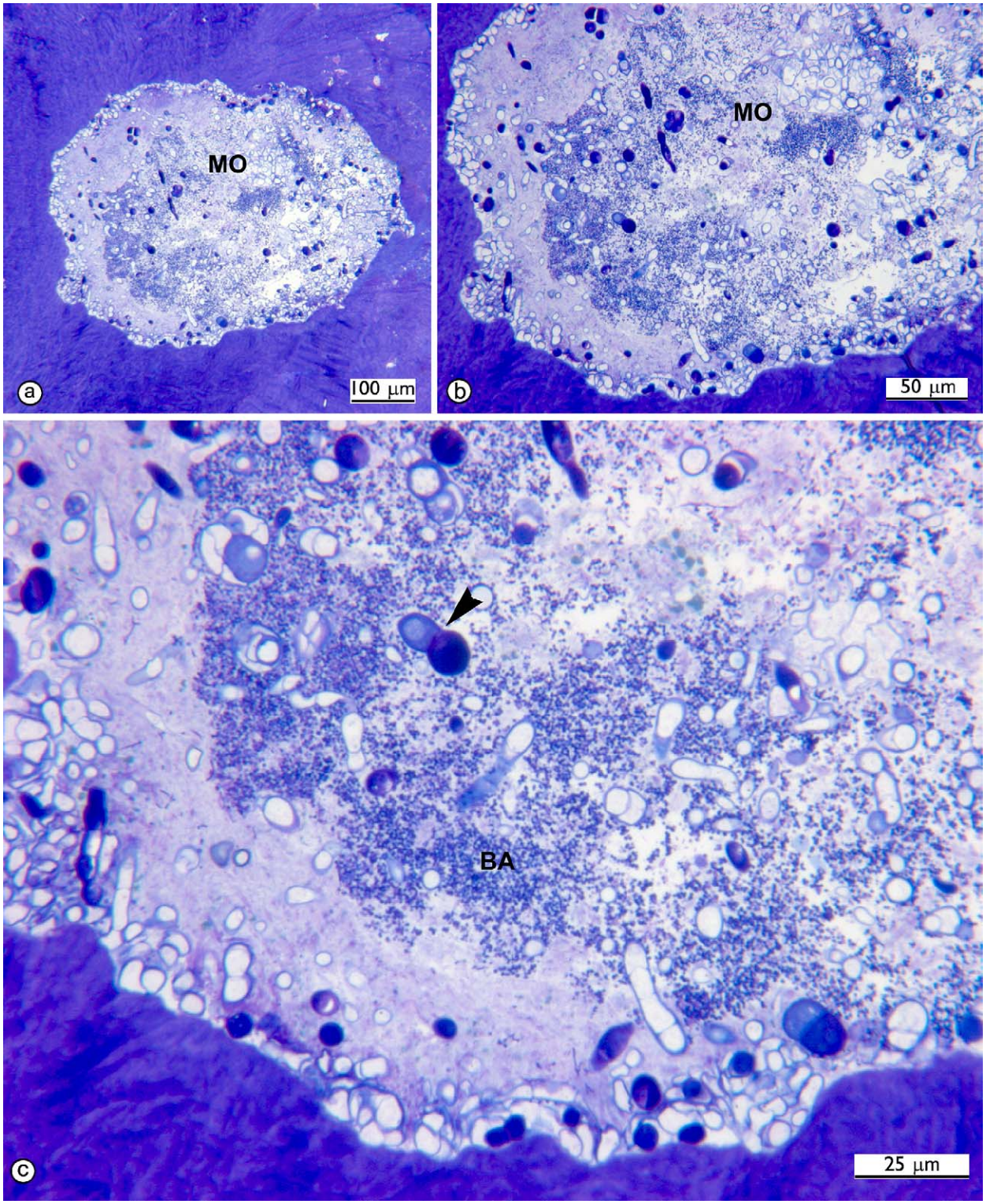

Fig 12. Stagewise magnifications of the accessory canal (AC) on the right side of Fig 11, **B**. The canal is filled with microorganisms (MO) of varying morphological forms. Note the small dot-like bacteria (BA in **C**) and the large pleomorphic organisms showing filamentous and dividing forms (arrowhead), which seem to be yeast stage of fungi. Original magnifications: **A**, ×100; **B**, ×280; **C**, ×800.

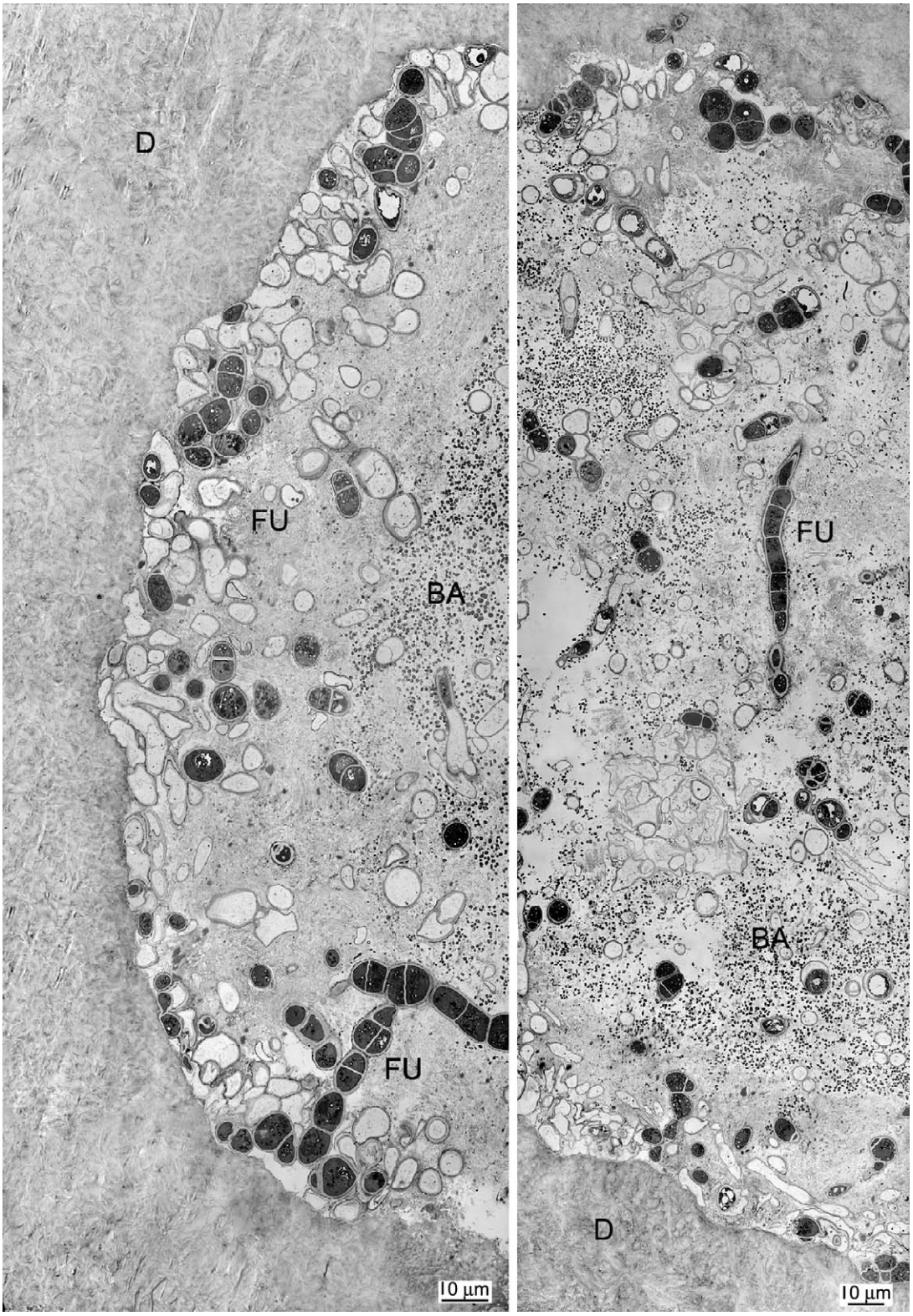

Fig 13. Composite transmission electron micrographs show the presence of bacteria (BA) and fungi (FU) in the accessory canal illustrated in Fig 12. Note the distinct electron lucent cell wall and the larger size of fungal organisms in comparison to that of the bacteria. The fungus shows several dividing forms, some of which form chains. The organisms marked FU are further magnified in Fig 14. Original magnifications: **A**, ×750; **B**, ×600.

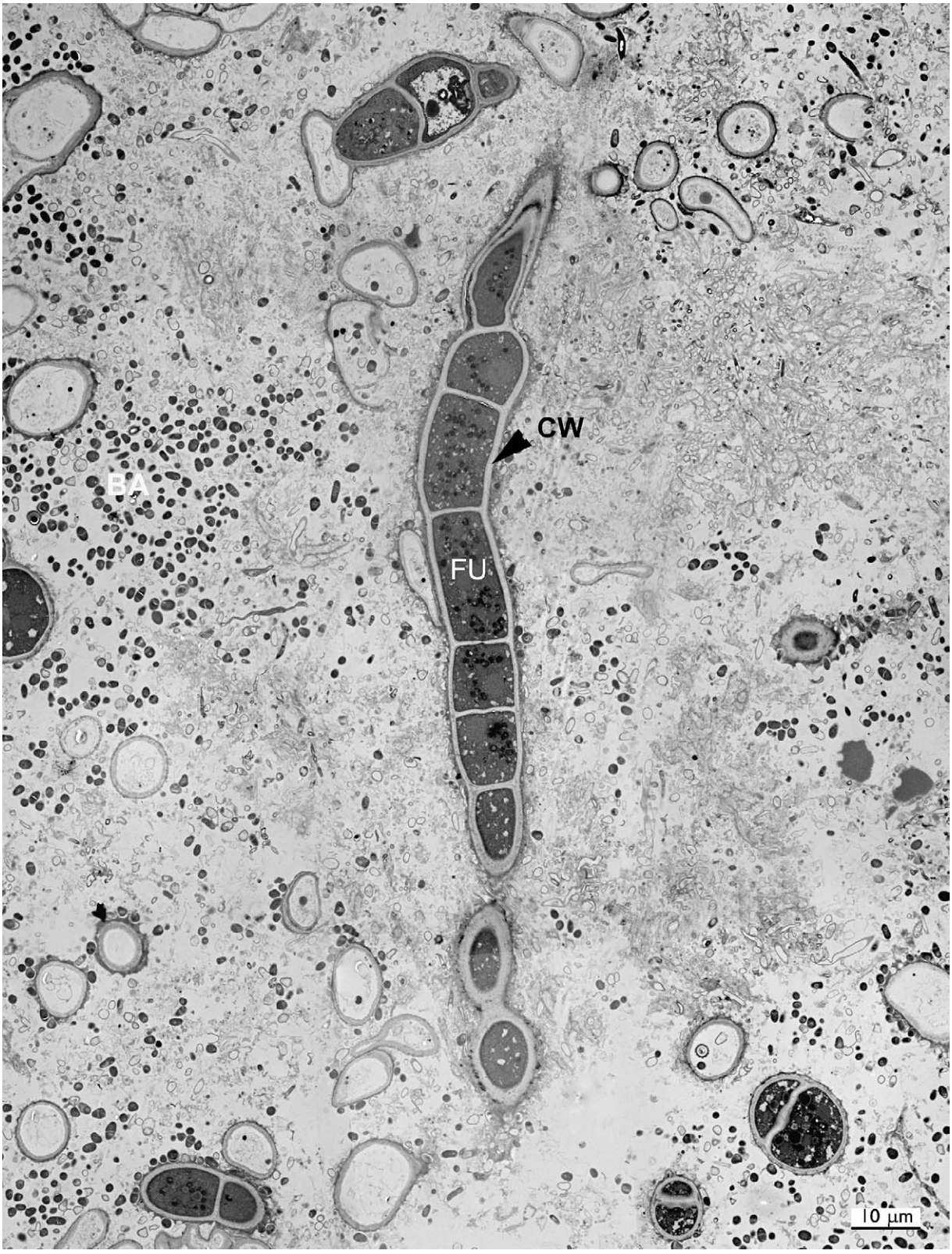

Fig 14. Higher magnification electron micrograph of the chain of large microorganisms (FU) in Fig 13, **B**. Note the much smaller size of the bacteria (BA) that consist of cocci, rods, and filaments. CW = cell wall. Original magnification ×1200.

bacteriological status of the treated canals. These studies showed that mechanical instrumentation and irrigation with physiological saline resulted in 100-fold to 1000-fold reduction of microbes from the root canal.[19] In the study,[19] however, none of the 15 root canal–treated and sampled teeth gave negative microbial cultures after the first appointment of treatment, which is fully in confirmation with the results of the present study. Even after 5 appointments and only mechanical instrumentation and saline irrigation, bacteria could be cultivated in 50% of the canals.[19] Other researchers[13] reported that 10 of the 23 (43%) root canal–treated teeth produced no microbial growth immediately after extensive instrumentation and irrigation with saline, a finding in discord with that reported here. Further reduction of intracanal viable microbes can be achieved by the application of tissue lytic and antimicrobial agents such as NaOCl alone[20] or NaOCl and EDTA in sequence.[21] This series of studies also showed the importance of application of bactericidal intracanal medicaments during the treatment.[22,52]

The efficiency of reducing intracanal microbes by instrumentation with NiTi devices in comparison to that of stainless steel hand files has been investigated[24,25] by adopting methods "similar to the series performed by Byström et al."[19-22] In one study,[24] it was found that only 13 of the 46 (28%) teeth sampled were culture-negative after instrumentation and saline irrigation. There was no statistical difference between the outcomes of instrumentation with stainless steel hand files and NiTi rotary instruments. In the other investigation,[25] it was shown that addition of rinsing with NaOCl solution resulted in better antibacterial effect when the instrumentation exceeded ISO size #30-35. It was concluded that a predictable elimination of microorganisms from the canal system cannot be achieved by NiTi instruments irrespective of the size of the final instrument used. Application of iodine-potassium-iodide (IKI) as a short-time dressing during one-visit treatment has shown some promising results in terms of reducing intracanal microbes. However, 15 of the 52 (29%) teeth that were treated in one visit, with IKI short-time dressing, still contained cultivable microbes posttreatment.[53] In this context, it has to be pointed out that root canals showing negative microbial cultures do not imply that the entire canal system of the teeth involved are "rendered bacteria free" as sometimes described.[15,25] Rather, there were no microorganisms recoverable when an advanced bacteriological method was used. This is because the microbial samples in these investigations were obtained from the instrumented main canal(s) only. It is obvious from the study reported here that there are root canal regions harboring microorganisms that cannot be reached by such sampling procedures.

It has also been recommended by others[54-56] to complete endodontic treatment of nonvital teeth with infected root canals in 1 session, without any intracanal microbicidal dressing. Intraradicular microbes surviving root canal treatment are argued to be entombed by obturation of the root canal and die off as a result of inadequate nutrients.[55] "These microbes may no longer interfere with the periapical healing process."[56] The long-standing popular notion of entombment and perishing of intraradicular microbes posttreatment still awaits scientific validity, but in the light of contemporary understanding of apical periodontitis as an intraradicular biofilm-induced chronic disease would be a challenging task to realize. It has been shown that periapical healing of some teeth occurs even when microbes are present in the canals at the time of obturation.[18] Although this may imply that the organisms may not survive posttreatment, it is more likely that the microbes may be present in quantities and virulence that may be subcritical to sustain the inflammation of the periapex, or that they remain in a location where they cannot communicate with the periapical tissues.

Taken together, there is substantial evidence[19-25] that negative microbial cultures are consistently obtained only after adequate instrumentation, rinsing of the canals with NaOCl and EDTA, and application of an intracanal microbicidal dressing. Short intra-appointment application of a bactericidal dressing has not so far resulted in a satisfactory reduction of root canal microbes.[52,53] Further, certain sessile bacteria protected in biofilms have been found to be more than 1000 times resistant to antimicrobial agents as the same organisms in planktonic form.[57,58] Therefore, additional caution is necessary about the positive antimicrobial effects of potential root canal disinfectants reported based on short-term in vitro experiments.[59] Purely based on biological considerations, a treatment plan involving meticulous instrumentation, irrigation with NaOCl, rinsing with EDTA, and application of a microbicidal dressing for a sufficient duration of time to be effective, cannot be completed in one treatment session with contemporary technology. Therefore, the results of this study do not provide the biological basis for treating teeth with infected necrotic pulps in one visit. On the contrary, they provide strong histomorphological evidence in support of following a treatment protocol based on carefully controlled investigations that consistently give negative microbial cultures of the treated canals.[19-25]

CONCLUSIONS

Fourteen of the 16 instrumented and root canal–treated mandibular molars showed residual infection of mesial roots after instrumentation, irrigation with NaOCl, and obturation were completed in a one-visit

treatment. The infectious agents were mostly located in the uninstrumented recesses of the main canals, the isthmus communicating between them, and in accessory canals. The microbes in these untouched locations existed primarily as biofilms that were not removed by instrumentation and irrigation with NaOCl in 1 treatment session. In view of the great anatomical complexity of the root canal system, particularly of molars,[26,27] and the ecological organization of the flora into protected sessile biofilms[5,47] composed of microbial cells embedded in a hydrated exopolysaccharide-complex in microcolonies,[4] it is very unlikely that an absolutely microorganism-free canal system can be achieved by any of the contemporary root canal preparation, cleaning, and root-filling procedures, particularly in 1 treatment session. These findings highlight the importance and necessity of stringently applying non-antibiotic chemomechanical measures in order to disrupt the biofilms and reduce the intraradicular microbial load to the lowest possible level to ensure the most favorable long-term prognosis for treatment of infected root canals.

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