Carbohydrate Research, 198 (1990) 331–341
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

In vivo DEGRADATION OF OXIDIZED, REGENERATED CELLULOSE

S. DAN DIMITRIJEVICH*, MATTHEW TATARKO*, ROBERT W. GRACY*†, GARY E. WISE‡, LAWRENCE X. OAKFORD‡,

**Tissue Repair Unit, Department of Biochemistry, ‡Department of Anatomy, The University of North Texas, Texas College of Osteopathic Medicine, Forth Worth, TX 76107, (U.S.A.)*

CARY B. LINSKY, AND LOLA KAMP
Johnson & Johnson Patient Care, Inc., New Brunswick, N.J. 08903, (U.S.A.)

(Received June 26th, 1989; accepted in revised form, September 27th, 1989)

ABSTRACT

Oxidized, regenerated cellulose (ORC) was surgically implanted on the uterine horns of rabbits, and its biodegradation was studied *in vivo*. Samples of peritoneal lavages, serum, and urine were collected during the degradation process and analyzed for carbohydrate components utilizing high-performance liquid chromatography with pulsed amperometric detection (h.p.l.c.–p.a.d.). Degradation was rapid, and oligomeric products were evident primarily in the peritoneal fluid from the implantation site, with no apparent accumulation in either the serum or the urine. The size distribution and the amount of the oligomeric products decreased after day one, and by day four peritoneal lavages were essentially free of oligomers. The structure of the products formed was consistent with the lability of the polymer in solution, and the kinetics of degradation paralleled the results of the previously reported *in vitro* studies. Rabbit peritoneal macrophages, when incubated with ORC *in vitro* were observed to readily ingest and hydrolyze the polymeric material. A mechanism of degradation consisting of chemical depolymerization, followed by enzymatic hydrolysis mediated by glycosidases endogenous to peritoneal macrophages, is proposed.

INTRODUCTION

Interceed® (TC7), an oxidized, regenerated cellulose (ORC) adhesion barrier, has been demonstrated to prevent post-surgical tissue adhesions[1]. In addition to the desirable mechanical properties of strength and flexibility, ORC is biodegradable. Previous studies from this laboratory[2] using ^{13}C-F.t.-n.m.r. and F.t.-i.r. spectroscopy, scanning and transmission electron microscopy (s.e.m. and t.e.m.), and direct chemical analyses, have shown that the polymer contains a heterogeneous distribution of carboxylic groups at C-6 and carbonyl groups at C-2 or C-3. When ORC is incubated at physiological pH and temperature, simulating

†To whom correspondence should be addressed.

0008-6215/90/$ 03.50

its *in vivo* application, the material is solubilized by β-elimination initiated depolymerization generating oligosaccharide and monosaccharide components[3]. *In vitro* studies also showed that incubation of ORC in serum results in more extensive degradation to smaller oligomers and monosaccharides than incubation in phosphate-buffered saline (PBS). The degradation of ORC *in vitro* was evaluated by high-performance liquid chromatography (h.p.l.c.) using triple pulse amperometry (pulsed amperometric detection—p.a.d.) of the underivatized carbohydrate products[4,5]. *In vivo* studies of periodate-oxidized cellulose, using ^{14}C-labeled material, presented a qualitative picture of biodegradation to smaller metabolizable fragments[6]. The experimental approach for the *in vivo* studies described herein was to implant rabbits with Interceed® and to examine their body fluids for degradation products using h.p.l.c.–p.a.d. Samples were obtained as lavages from the peritoneal cavity surrounding the implant site, as well as from urine and blood. The h.p.l.c.–p.a.d. profiles were compared with those obtained from sham-operated animals which were not implanted. The purpose of this study was to determine the nature of the products of the biodegradation of ORC *in vivo*, and to examine the distribution of these degradation products in the animal during the post-surgical period.

RESULTS

Fig. 1 shows h.p.l.c.–p.a.d. analyses of peritoneal lavages from ORC-implanted animals and the corresponding sham-operated controls after four and eight hours post surgery. The appearance of oligomers (the elution time of which increases with their size and charge) is detected in the lavages from ORC-implanted animals only. Also significant is the increase in the amounts of early eluting components (*e.g.*, D-glucuronic acid) observed at eight hours following implant, when compared with those present in the samples four hours post implant. Fig. 2 shows the kinetics of the formation of oligomers and their subsequent degradation to smaller fragments during the four-day period following ORC implantation. By day four, the elution profile of lavages from test animals is essentially the same as that for the controls presented in Fig. 1. Since ORC is a heterogeneous polymer, consisting of both D-glucuronic acid and D-glucose units, chromatographic standards are not available for a detailed identification of the oligomers arising from its depolymerization. The majority of oligoglycosiduronates arising from the biodegradation process were found in the degree of polymerization (d.p.) 2 to d.p. 7 region. This retention region approximately 9–30 min, was defined using a partial enzymatic hydrolysate of poly-D-galacturonic acid, D-glucose, cellosiose, and D-glucuronic acid, and the oligomers obtained from the enzymatic hydrolysis of alginic acid. The use of these standards was described in a previous publication concerned with *in vitro* biodegradation of ORC[3], and the retention times for oligogalactosiduronates are indicated by arrows in Fig. 2. These data are consistent with the macroscopic evidence obtained by visual observations of the test animals and the controls

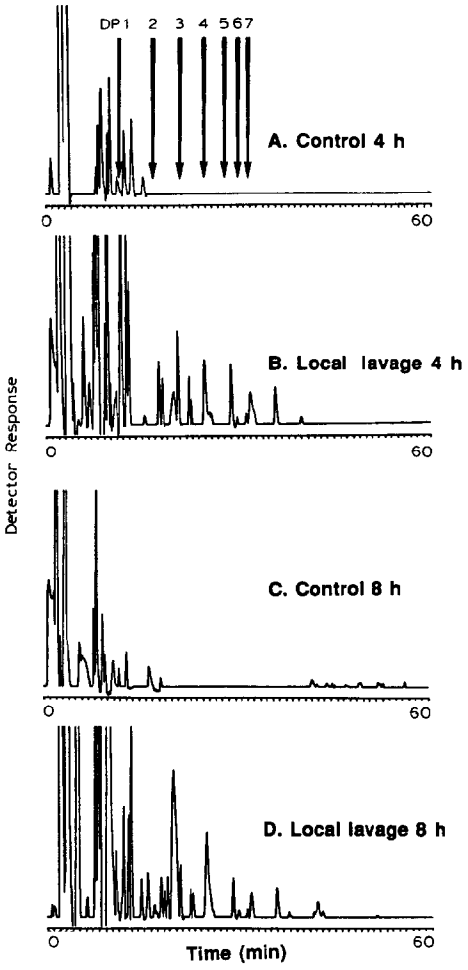

Fig. 1. H.p.l.c.–p.a.d. analyses of peritoneal lavages from test (A,C) and control (B,D) animals. Samples were obtained 4 h (A) and 8 h (C) after implantation of animals with ORC. Controls were sham operated and sampled at 4 h (B) and 8 h (D). Chromatographic analysis, described in the experimental section, shows appearance and size/charge distribution of monomeric and oligomeric entities. Retention times for D-glucose, cellobiose, and D-glucuronic acid under these analytical conditions were 3, 3.8, and 12.6 min, respectively.

previously recorded[7]. Table I shows that the material gels and is then solubilized in the peritoneal cavity during a time period similar to that discussed above.

Analysis of the serum from the controls and the test animals, obtained during the time period of the study (four days), showed no changes in the region of the chromatographic profiles corresponding to the oligomers found in the peritoneal fluid samples. Significant differences in the concentrations of the fast-eluting components, such as D-glucose or D-glucuronic acid, were difficult to measure because of the interference due to serum glucose. Similarly, the analysis of samples taken

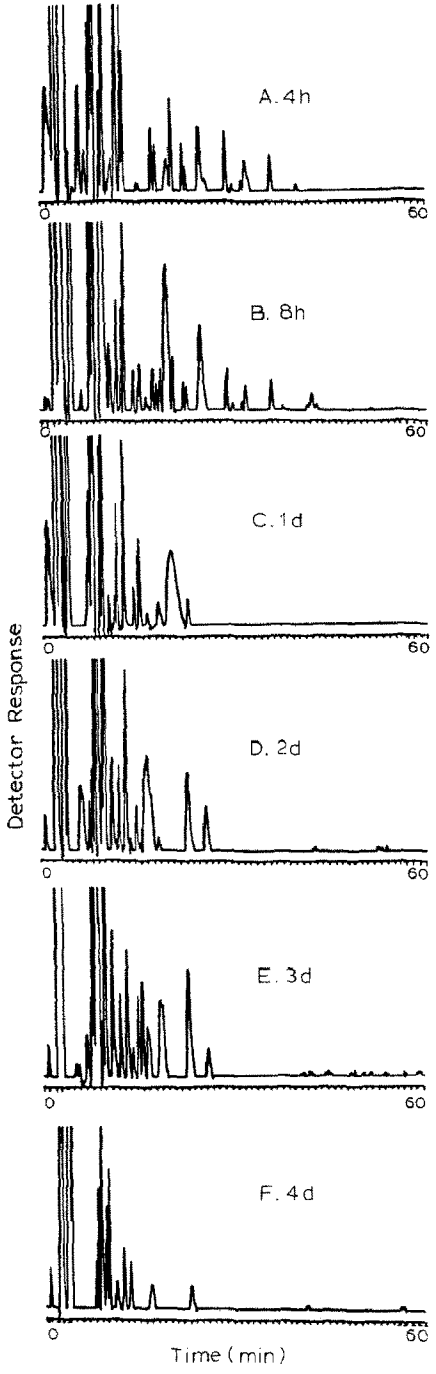

Fig. 2. Kinetics of biodegradation of ORC *in vivo*. Lavages were obtained from test animals at 4 h (A), 8 h (B), 1 d (C), 2 d (D), 3 d (E), and 4 d (F). Analyses of the samples show appearance of oligomers and their clearance.

TABLE I

PHYSICAL APPEARANCE OF ORC AFTER IMPLANTATION INTO RABBIT PERITONEUM

Time post implantation	*Appearance*
0.17 h	Similar to original material
0.50 h	Similar to original material
1 h	Similar to original material
2 h	Yellowish brown
4 h	Dark brown
8 h	Very gelatinous knit fabric; fragile, rips easily
24 h	Completely gelatinous; not fabric-like
48 h	Only small fragments of gelatinous material remain

TABLE II

COMPARISON OF DEGRADATION PRODUCTS OF ORC *in vivo* AND *in vitro*[a]

Elution time (min)	*D.p.*[b]	*In vitro*[c]		*In vivo*	
		PBS	*Serum*	*2 Days*	*4 Days*
0–13	1	9	90	90	98
13–36	2–10	69	7	10	2
>36	>10	22	3	0	0

[a]Values are percentages from integration of areas under chromatographic elution profiles. [b]Degree of polymerization (d.p.) determined from oligo-D-galactosiduronates. [c]*In vitro* data summarized from studies reported in reference 3.

directly from the bladder of the animals, or from voided urine, showed indistinguishable differences between the test group and the controls.

Figs. 3–6 show transmission and scanning electron micrographs(s) of rabbit peritoneal macrophages, which have been incubated with the lead acetate salt of Interceed® (ORC). Fig. 3 shows a macrophage attached to the lead-labelled ORC with several regions where endocytosis of the electron-dense material appears to be taking place. Another macrophage (Fig. 4) displays numerous endocytic vacuoles filled with the ORC, as well as images of its engulfment. In some instances, one can find macrophages in which some of the lead appears to have dissociated from the Interceed® in the endocytic vacuole (probably a secondary lysosome at this stage) and is now found associated with membranes of the nuclear envelope, rough endoplasmic reticulum, and mitochondria (Figs. 4 and 5).

Previous studies have shown that when ORC is perfused with heavy-metal salts such as lead acetate, its physical integrity is retained, and the D-glucuronic acid residues participate in complex salt formation[2]. To further demonstrate that the resulting complex salt is endocytosed by the macrophages, the latter were

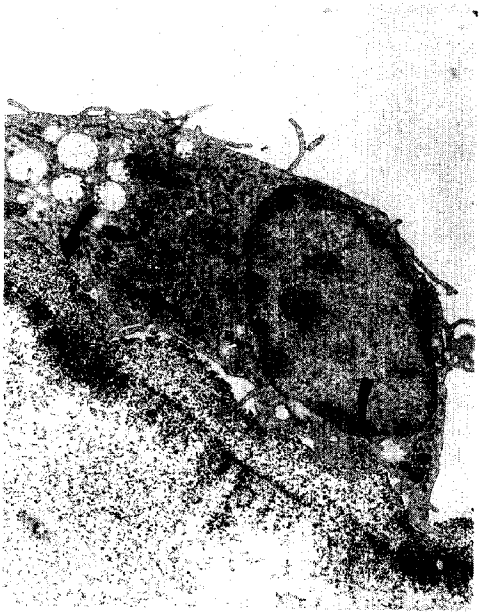

Fig. 3. Transmission electron Micrograph of a rabbit peritoneal macrophage after incubation with powdered lead-labelled ORC. Note the attachment of the cell to a large fragment of labelled ORC, and regions (arrows) where the macrophage may be endocytosing the ORC.

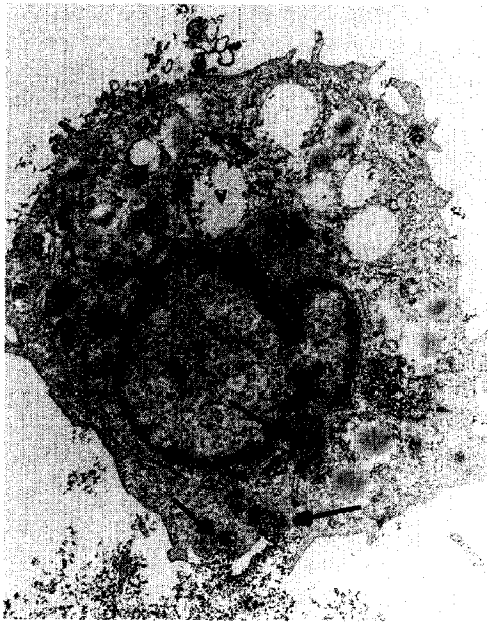

Fig. 4. T.e.m. of another macrophage showing endocytic vacuoles (V) containing the lead-labelled ORC (arrows). Fragments of ORC which are being phagocytosed are clearly visible (arrows) outside the vacuoles.

Fig. 5. High-power t.e.m. of a portion of a macrophage showing some lead (arrows) remaining in secondary lysosomes. Other lead ions apparently dissociate from the ORC as it is digested and they become associated with membranes of the nuclear envelope (NE) and rough endoplasmic reticulum (ER).

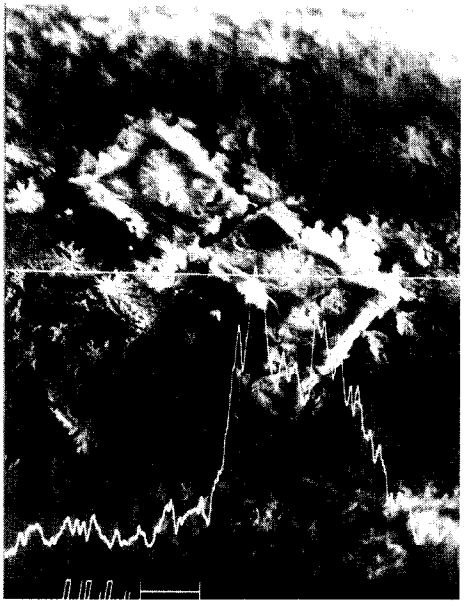

Fig. 6. Scanning Electron Micrograph of a macrophage that had been incubated with powdered lead-labelled ORC prior to being ashed. The X-ray emission scan, set to detect lead, was conducted at the level of the horizontal white line, and the scan profile shows the high level of lead within the macrophage, indicating that the lead-labelled ORC had been endocytosed. Bar = 10 μm.

ashed, and an elemental analysis of the cells was conducted. Under the scanning electron microscope, X-ray emission revealed a high content of lead in the cells in comparison to the low levels present in the extracellular spaces (Fig. 6).

DISCUSSION

The studies of the degradation of ORC using h.p.l.c.–p.a.d. analysis of the products present in the peritoneal lavages of the test animals provide definitive data on the *in vivo* behavior of oxidized, regenerated celluloses. The chromatographic profiles of degradation products are consistent with the structural features of the polymer and the proposed mechanism of *in vitro* degradation, the details of which have been previously reported[3]. Furthermore this course of events is supported by the macroscopic observations of the physical behavior of Interceed® *in vivo* (Table I) with respect to the chronology of hydration, gelling, and clearance. Table II compares h.p.l.c.–p.a.d. profiles of the biodegradation products resulting from the *in vitro* and the *in vivo* studies. Thus *in vivo*, the sizes and the amounts of the oligomeric products began to decrease during day one, and by day four post implant, lavage profiles were essentially free of the oligomers. Time-dependent increase in the extent of degradation of oligomers *in vitro*, in serum, parallels the above *in vivo* results as shown by the similarity between their h.p.l.c. profiles.

The analysis of the serum and urine samples from the test animals showed no accumulation of biodegradation products (oligomers), which were detected in the peritoneal fluid of the test animals. We have previously reported the *in vitro* biodegradation of ORC in serum[3], and the approximate quantitation of the oligomers produced is shown in Table II. The quantitation of monomeric units produced in serum and urine is difficult due to the interferences in that region of the elution profile (*e.g.*, glucose in serum and salts in urine). In the present *in vivo* study, the absence of oligomers from the serum and urine of the test animals is unlikely to be due to the detection limitations of h.p.l.c.–p.a.d. technique strongly suggesting involvement of a predominantly local, rather than systemic mechanism of clearance of Interceed®.

Peritoneal macrophages are normally elicited in response to trauma of peritoneal structures and their rapid accumulation at the site of implantation of ORC has been observed[8]. Also during morphological and histopathological studies of Surgicel®, an oxidized cellulose material related to Interceed®, involvement of macrophages in its biodegradation was demonstrated[9]. Since it is well established that an important function of the macrophages is to recognize and remove non-endogenous entities from the system via endocytosis, our attention was drawn to the response of these elements of the immune system to the invasive events under discussion. The intracellular digestion of such materials is carried out by a wide variety of resident hydrolytic enzymes, including β-D-glycosidase and β-D-glucosiduronase[10]. In order to demonstrate the participation of peritoneal macrophages in the biodegradation of ORC, we took advantage of the reaction of D-

glucuronic acid residues in ORC with lead acetate, to give a complex lead salt, which became visible by X-ray emission during s.e.m., as well as in thin sections during t.e.m. Thus, incubation of lead acetate labelled ORC with rabbit peritoneal macrophages, followed by s.e.m. examination showed that, indeed, the ORC (as detected by elemental analysis of lead) is taken up by the macrophages. T.e.m. revealed that the macrophages attach to and appear to endocytose the ORC so that numerous endocytic vacuoles containing the complex lead salt are seen within the cells, as well as images of the macrophages appearing to engulf the adjacent ORC. This phase of clearance of ORC complements the hydration/gelling and subsequent chemical depolymerization processes. Thus a mechanism of clearance which includes a rapid and localized macrophage-assisted biodegradation is preferred over a slower systemic process.

In conclusion, the results of the present *in vivo* studies follow very closely the course of degradation predicted on the basis of the *in vitro* studies[3] and macroscopic morphological observations previously obtained[7]. Both are related to the inherent structural instability of ORC in the physiological environment. The dynamic aspect of the *in vivo* system assists in a rapid local clearance of the degradation products, and the buildup of significant systemic levels is not detectable in either serum or the urine.

Finally, it is important to point out that h.p.l.c.–p.a.d. analysis of biological fluids can be developed into an excellent sensitive and quantitative method for evaluating carbohydrate metabolism *in vivo*. The use of h.p.l.c.–p.a.d., while a relatively new tool for carbohydrate analyses, has initially been applied, primarily, to *in vitro* problems. The fact that biological materials, such as peritoneal lavages, serum, and urine, were relatively free of interfering components under the conditions used for the detection of the oligomers, suggests an increasing potential for the use of h.p.l.c.–p.a.d. in examination of complex biological materials without the derivatisation of the carbohydrate components.

EXPERIMENTAL

Materials. — ORC, designated Interceed®, was obtained from Johnson & Johnson Patient Care. All chromatographic supplies and reagents were from Dionex Corporation.

Implantation of ORC. — A 5.08 × 7.62-cm piece of Interceed® was applied to each uterine horn of 3–4 kg rabbits. The surgical procedures used for laparotony were identical to those described in the prior report[1]. Sham surgery control animals were treated identically, except that Interceed® was not implanted.

Recovery of body fluid samples. — Blood was recovered via the ear vein, allowed to clot, and the serum was obtained after centrifugation. Urine was collected from the animals housed in metabolic cages. After sacrifice of the animals, the bladder contents were also recovered by bladder puncture. Local lavage samples were obtained immediately after sacrificing the animals. The uterine horns

were then carefully lifted *in situ* and rinsed with 5 mL of sodium chloride. All exogenous material present was gently scraped into the wash. The same 5-mL sample was used to wash the horns a second time. All fluids were frozen and stored in polypropylene centrifuge tubes at −20°.

H.p.l.c.-p.a.d. analyses of body fluids. — Samples were thawed and centrifuged through Amicon 30K molecular weight cutoff membranes. All separations were carried out on a Dionex AS6 pellicular anion exchange column (4.6 × 250 mm) with an AG6 guard column. Samples (50 μL) were eluted by one of two different elution methods optimized for the separation of either oligomers or monomers of the saccharide moieties of the original materials. Oligomers were eluted with a linear gradient of 100% A (5mM NaOH and 10mM sodium acetate) to 10% A and 90% B (5mM NaOH and M sodium acetate) in 35 min, followed by an isocratic elution for an additional 25 min. Monomers were resolved with a linear gradient of 100% C (5mM NaOH) to 100% eluant D (150mM NaOH containing 500mM sodium acetate) in 30 min, which was then eluted isocratically for an additional 15 min. Flow rates were 1.0mL.min^{-1}, and all analyses were followed by a wash of 700mM NaOH at a flow rate of 0.5 mL.min^{-1} to improve baseline stability. Detection was accomplished with a Dionex PAD 2 detector with a gold working electrode operated in the triphasic mode (E_t = 0.05 V for 360 ms, E_2 = 0.80 V for 120 ms and E_3 = 60 V for 420 ms) at 300 nA full scale. Assignment of degree of polymerization (d.p.) was based on chromatographic analysis of commercially available standards and oligomers obtained by enzymatic hydrolysis of poly-D-galacturonic acid and alginic acid. The latter, characterized by f.a.b.-m.s., included: a) (1→4)-β-tri- and penta-D-mannuronic acid with a 4,5-unsaturated non-reducing end unit, b) (1→4)-β-di-, tri-, tetra-, and penta-D-galacturonic acid with a 4,5-unsaturated non-reducing end unit, and c) (1→4)-β-di-, tetra-, and hexa-D-galacturonic acid. These were kindly provided by Dr. James F. Preston III, Department of Microbiology and Cell Science, the University of Florida, Gainsville, FL. Data collection, manipulations, and baseline corrections were carried out using a MacIntosh SE microcomputer and Dynamax Complete Data and Control Software.

Macrophage isolation. — Macrophages from adult female New Zealand white rabbits (3–4 kg) were induced by interperitoneal injection of Difco thioglycolate medium (200 mL). After 4 d the rabbits were sacrificed, and the macrophages were removed by lavaging the peritoneal cavity with PBS (250 mL) containing 10 U.mL^{-1} of heparin sulfate. After three additional washes with the above buffer, the washes were combined with the original lavage and centrifuged (800*g* for 10 min). The erythrocytes were removed by ficol-hypaque centrifugation (400*g* for 30 min), and the macrophages were isolated and resuspended in RPMI 1640 media supplemented with gentamycin (150 μg.mL^{-1}) and amphotericin B (2.5 μg. mL^{-1}). The cells were then incubated for 45 min at 37° with powdered lead-labelled ORC, prepared by perfusing the woven gauze with lead acetate, and the cells were then subjected to electron microscopy.

Transmission and Scanning electron microscopy (t.e.m. and s.e.m.). — In

order to prepare samples for t.e.m., the macrophages and their substrate, following the incubation with the lead acetate labelled ORC, were fixed in glutaraldehyde (2% in 0.1M cacodylate buffer) for 1 h. The samples were then rinsed in buffer and post-fixed in OsO_4 (1% in distilled H_2O) for 1 h. Dehydration of the samples was carried out by rinsing through a series of aqueous ethanol mixtures containing an increasing amount of alcohol (10%, 30%, 50%, 70%, 95%, and 3 × 100%), followed by washing in propylene oxide. The samples were then embedded in Epon, and thin sections were cut on a Porter Blum-MT2B ultramicrotome, and, after staining, were examined on an Hitachi H-600 electron microscope.

For s.e.m., a sample of macrophages was removed from the ORC substrate, dried down on a glass coverslip and ashed for 0.5 h in a 300° oven. The sample was then placed on an aluminum stub and examined with an ETEC Autoscan scanning electron microscope utilizing a Tracor Northern TN2000 X-ray probe to detect the X-rays emitted from the lead after being struck by the electron beam.

ACKNOWLEDGMENTS

This work was supported in part by research grants from the National Institutes of Health (AG01274), The Robert A. Welch Foundation (B-0502), The Texas Advanced Technology and Research Program (#2147 Molecular Basis of Impaired Wound Healing in Aging), and by Johnson & Johnson Patient Care, Inc.

REFERENCES

1 (a) C. B. LINSKY, M. P. DIAMOND, T. CUNNINGHAM, B. CONSTANTINE, A. H. DECHERRY, AND G. S. DI ZEREGA, *J. Reprod. Med.*, 32 (1987) 17–20; (b) M. P. DIAMOND, C. B. LINSKY, T. CUNNINGHAM, B. CONSTANTINE, G. S. DI ZEREGA, AND A. H. DECHERNEY, *Microsurgery*, 8 (1987) 197–200.
2 S. D. DIMITRIJEVICH, M. TATARKO, AND R. W. GRACY, unpublished results.
3 S. D. DEMITRIJEVICH, M. TATARKO, R. W. GRACY, C. B. LINSKY, AND C. OLSEN, *Carbohydr. Res.*, in press.
4 R. D. ROCKLIN AND C. A. POHL, *J. Liq. Chromatogr.*, 6 (1983) 1577–1590.
5 L. M. CHEN, M. G. YET, AND M. C. SHAO, *FASEB J.*, 2 (1988) 2819–2824.
6 M. SINGH, A. R. RAY, AND P. VASUDEVAN, *Biomaterials*, 3 (1982) 16–20.
7 C. B. LINSKY AND L. KAMP, unpublished results.
8 F. MCCONNELL AND C. B. LINSKY, unpublished results.
9 A. M. PIERCE, O. W. WIEBKIN, AND D. F. WILSON, *J. Oral. Pathol.*, 13 (19829 661–670.
10 (a) S. GORDON, *Fed. Proc.*, 37 (1978) 7754–7758; (b) R. C. PAGE, P. DAVIES, AND A. C. ALLISON, *Int. Rev. Cytol.*, 52 (1978) 119–157; (c) S. GORDON, J. TODD, AND Z. A. COHN, *J. Exp. Med.*, 139 (1974) 1228–1248.