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Gradient chamber

A new method for migratory fractionation of inflammatory response in vivo

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Summary. A new and simple method to study the inflammatory process in vivo using a diffusion-migration chamber is described. The influx of inflammatory elements into chambers was studied by light and electron microscopy. This gradient chamber (GC) was developed by substituting one of the filters of the classic diffusion chamber by a polyvinyl sponge cylinder. In the implanted chambers this plastic framework acts as a tridimensional gradient, through which the acute and chronic inflammatory processes migrate with different speeds and are thus divided into two distinct and widely separated waves. By delaying the overlapping of the first wave by the second, this bioassay allows a longer time for observation of the cell-matrix or cell-matrix-graft interaction during the first inflammatory wave and permits studies by methods such as immunocytochemistry, histochemistry or biochemical analysis.

Key words: Diffusion-migration chamber – Fractionation of inflammatory response in vivo

Introduction

The subcutaneous sponge implantation technique (for general references see Hølund et al. 1979) was first described by Grindlay and Waugh (1951). These authors implanted polyvinyl alcohol sponges in animals and observed the gradual filling of the intertices by the migrating connective tissue of the host. The method furnishes an endogenous site favourable for mesenchymal growth that minimises the difficulties and limitations of tissue culture (Boucek and Noble 1955) and has been used to evaluate neoformation and maturation of connective tissue in healthy (Edwards et al. 1957; Kelly 1962) and diseased animals (Bhuyan et al. 1972; Mesquita 1982). We observed that the elements of the acute and chronic phases of the inflammatory process, migrating through the large pores of implanted sponges with different speeds, created a tridimensional gradient in vivo. This prevented rapid overlapping of the inflammatory phases, separating the inflammatory response into two distinct waves. We therefore coupled a polyvinyl sponge with a diffusion chamber, the latter allowing the nourishment of optional target implants but preventing their contact with host cells (Algire et al. 1954), resulting in a diffusion-migration chamber. We describe this new method here and present data on a kinetic study commenting on some aspects of the migrating inflammatory waves. This method has previously been presented in abstract form (Mesquita 1985, 1986, 1987a).

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Material and methods

Eighteen outbreed female albino mice weighing 25–30 g received a standard diet and tapwater ad libitum.

Chambers were composed of two parts which were mounted together: Part I was constituted by a polyvinyl sponge cylinder (gradient sponge) of 12 mm diameter, with an approximate pore size of 500 μm and 2 mm height (Fig. 1A, a), made by cutting a 2 mm sponge sheet by means of a cork-cutter with 12 mm diameter. This represents the migration face of the chamber. Part II (Fig. 1A, e) was constituted by three lucite rings (Millipore Corp., New Bedford, Mass., P/R 1400) (Fig. 1A, b) glued with cement N° 1 (Millipore) (Fig. 1A, c), having interposed between two of them a membrane filter with 0.45 μm pores (Millipore) (Fig. 1A, d). This represents the diffusion face of the chamber. Part I was boiled for 1 h in a 5% aqueous solution of Extran MAO2-neutral (Merck S.A. Industrias Quimicas, RJ) and cleansed in running water for 4 h and then sterilized by boiling in distilled water for 2 h. Part II was sterilized by ultraviolet light for 24 h. Chambers were assembled

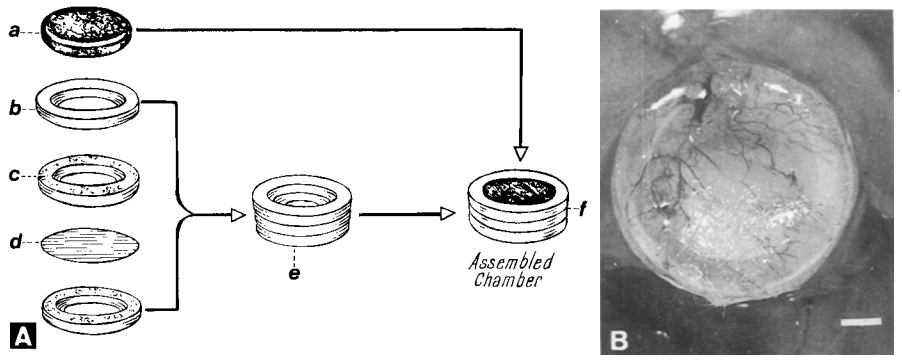

Fig. 1. (**A**) Components of gradient chamber: (**a**) Polyvinyl sponge, (**b**) lucite ring, (**c**) glue, (**d**) membrane filter, (**e**) open chamber, (**f**) assembled chamber. (**B**) Macroscopy. 4 days implanted GC. ×2. *Bar* = 2.5 mm

under aseptic conditions (Fig. 1 A, f), immediately before implantation.

One chamber was implanted subcutaneously in the back of each mouse, about 2 cm from the skin incision, using intraperitoneal Nembutal (50 mg/kg body weight) anesthesia, after the subcutaneous tissue had been dissected with scissors. Each group was selected at random and killed at 4, 7 and 14 days by ether overdose, and the chambers were rapidly trimmed from external adherent tissues. They were then opened and samples of each polyvinyl sponge and filter taken.

For paraffin embedding, samples were taken from each filter and polyvinyl sponge, fixed in modified Milloning's phosphate buffered formalin (Carson et al. 1973) for 48 h and processed as usual. Sections (5 μm) were stained with haematoxylin and eosin (HE), Lennert's Giemsa, Gomori's reticulin, periodic acid Schiff (PAS), Mallory's PTAH stain for fibrin, picrosirius-red method for collagen (Junqueira et al. 1979) and PAS-alcian blue pH 1 and 2.5 for sulfated and acid proteoglycans respectively. For plastic embedding, samples taken from filters and polyvinyl sponges were immersed in buffered 2.5% glutaraldehyde (1% cacodylate, pH 7.2) for 3 h. Thereafter, samples were rinsed and washed in buffered, postfixed for 2 h in ice cold 0.1 osmium tetroxide buffered in cacodylate, dehydrated in ascending concentrations of acetone and embedded in Epon. Semi-thin sections (0.1 μm) obtained on a Reichert Om U3 ultramicrotome were stained with 1% toluidine blue in 1% borax and methylene blue azure II-basic fuchsin and then examined under light microscopy. Suitable areas, selected for ultrathin sections, were double-stained with uranyl acetate and lead citrate and examined in a Zeiss EM 109 electron microscope at 60 Kv. For scanning electron microscopy (SEM), filters and cross-sectioned gradient sponges were fixed under similar technique employed for transmission electron microscopy (TEM), dehydrated in an alcohol gradient, mounted on studs to allow visualization of the internal surface of filters and the cross-section of sponges, dried to the critical-point and vacuum-coated with gold. Samples were examined in a Hitachi S-450 scanning electron microscope.

Results

Signs of suppuration were not seen in surgical sutures or implantation sites. On the 4th day, implanted chambers were covered by an adherent, thin and vascularized loose connective tissues (Fig. 1 B), which became progressively denser and thicker with time.

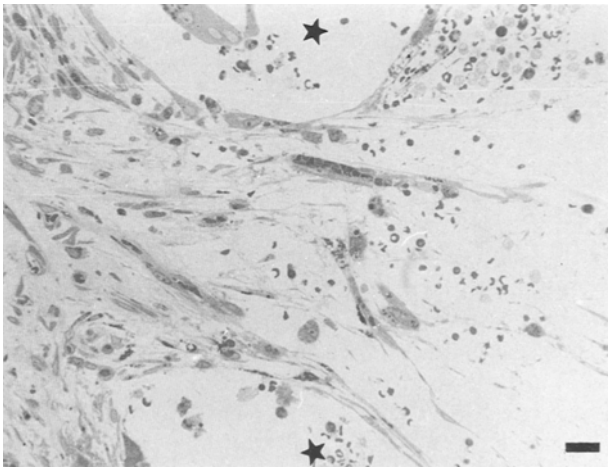

Fig. 2. The chronic wave crosses a sponge pore formed by a plastic mesh (★) overlapping the acute wave inside a 7 days implanted GC. Toluidine blue ×400. *Bar* = 10 μm

After implantation, gradient sponges were invaded by two successive and separate inflammatory waves. The acute phase already occupied the whole thickness of gradient sponges examined at 4 days and the chronic progressively overlapped the acute, having invaded the entire sponge by 14 days.

On light microscopy, the gradient sponge pores at 4 days were seen to be filled by a fine gel-fibrillary network, on which the cellular elements of the acute phase migrated. These elements, mainly represented by polymorphonuclear neutrophils (PMNs), monocytes and/or macrophages and erythrocytes, crossed the whole thickness of the migration sponge and had already reached the bottom of the chambers. Mean-while, the chronic phase of inflammation had started to migrate and some tips of a young highly vascular connective tissue, a granulation tissue principally formed by capillary sprouts, fibroblasts and histiocytes, had penetrated into the sponges' most peripheral pores. The extracellular matrix of the acute phase

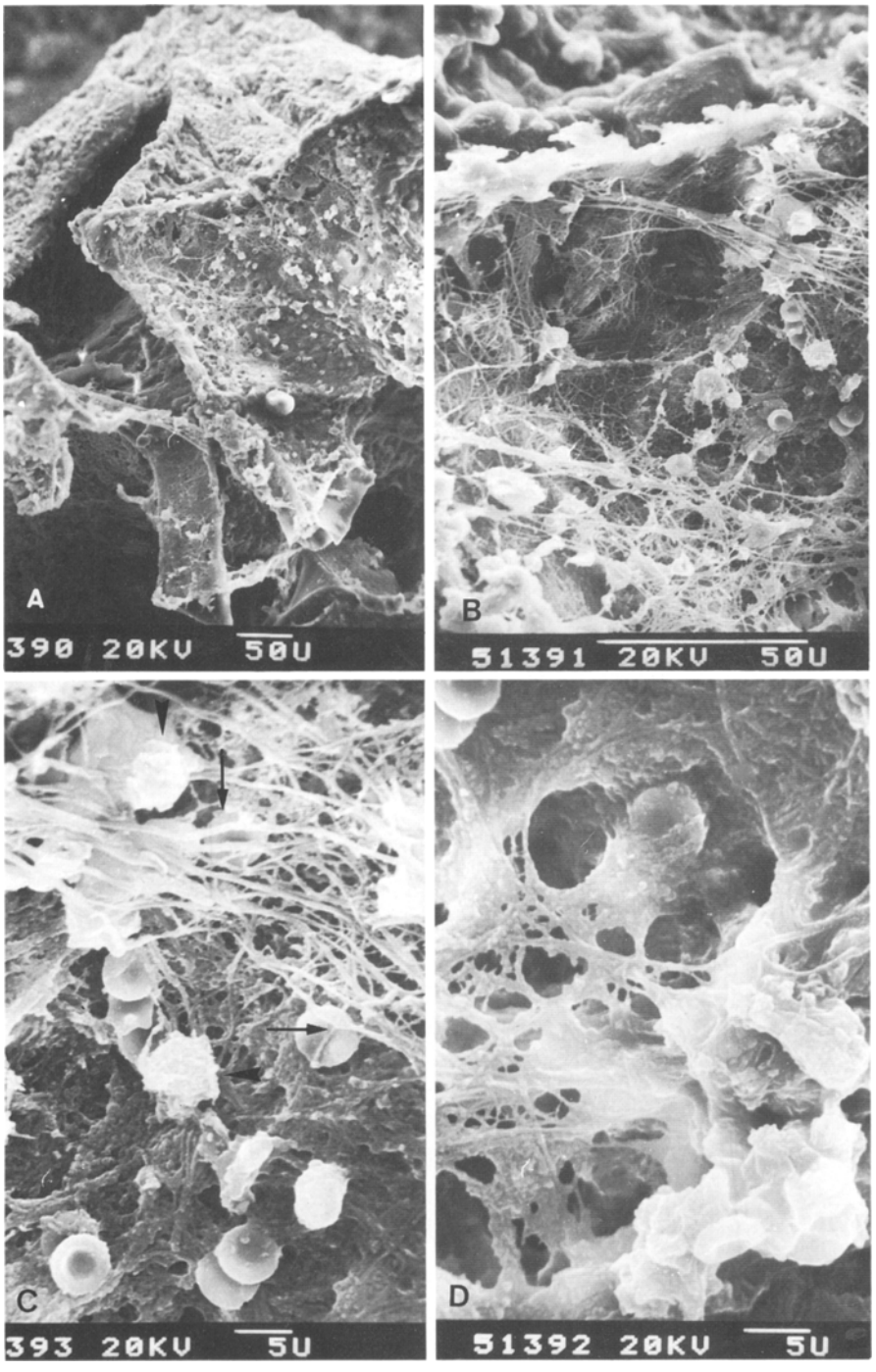

Fig. 3. (A–D). SEM. The migrated acute phase on the chamber bottom of a 7 days implanted GC. **(A)** The ECMA covering the plastic surfaces ($\times 140$. *Bar* = 50 μm). **(B)** form long fibrillar structures anchoring on the pore edges ($\times 580$. *Bar* = 50 μm). **(C)** support clambering migrating inflammatory cells (*arrowheads*), entrap erythrocytes (*arrows*) ($\times 1600$. *Bar* = 5 μm) and **(D)** build mazy-like pathways. $\times 2000$. *Bar* = 5 μm

(ECMA) stained positively for fibrin, glycoproteins, acid proteoglycans and very weakly for sulfated proteoglycans. Reticulin and collagen were negative.

By 7 days, the chronic inflammatory wave had invaded about one third of the thickness of the sponge, presenting two different patterns when the peripheral and internal pores of the GCs were examined. A relatively immature connective tissue filled the outer pores, where capillaries, fibroblasts and histiocytes predominated and a discrete granulomatous reaction around plastic meshes was observed. In the inner pores, on the front line of the second inflammatory wave, young granulation tissue similar to that observed invading the peripheral sponge pores on the 4th day overlapped the elements of the first inflammatory wave. Thin-walled capillaries, mainly supported by the fibrillar

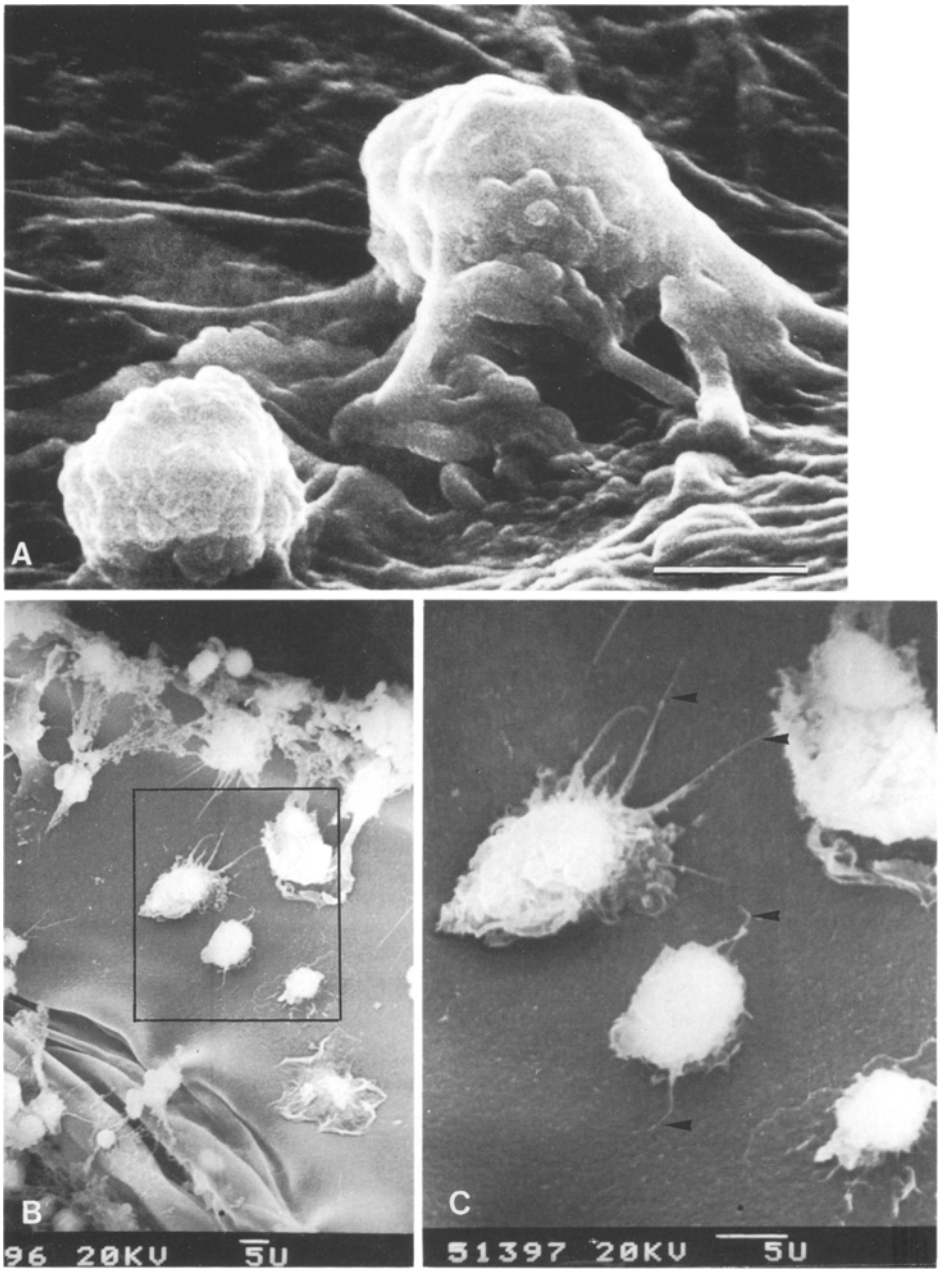

Fig. 4. (**A–C**). SEM. 7 days implanted GC. (**A**) Two cells migrating under the fine gel-fibrillar ECMA covering the sponge surface. × 4200. (**B**) The ECMA was hazardously torn off during the cross-sectioning of the gradient sponge, exposing migrating cells spreading on the plastic surface. × 800. (**C**) Magnification of area in **B**. Detail of filopodial extensions (*arrowheads*). × 2000. *Bars* = 5 μm

components of the ECMA, migrated as elongated hairpins often having lower shaped haemorrhages at their apices (Fig. 2). The extracellular matrix of the chronic phase (ECMC), was strongly positive for glycoproteins, acid and sulfated proteoglycans. Picrosirius-red stain, showed the presence of a delicate collagen fibril network of type I and III in the outer pores of the gradient sponges and scarce fine fibrils of type III in the inner pores when examined under polarized light. Reticulin apparently stained the same fibrillar structures as picrosirius-red. The elements of the acute phase occupied the inner two thirds of the sponges where the number of PMNs and monocytes and/or macrophages was increased. The ECMA showed an enhanced thickness of the fibrin network and a weakly increased stain positivity for glycoproteins and proteoglycans.

At 14 days, the second inflammatory wave had invaded the whole thickness of the gradient sponges and had already reached the inner surface of the filters. The peripheral granulation tissue was more fibroblastic and granulomatous around plastic meshes than at 7 days. Type III collagen fibrils were thicker and glycoproteins as well as proteoglycans stained more intensively.

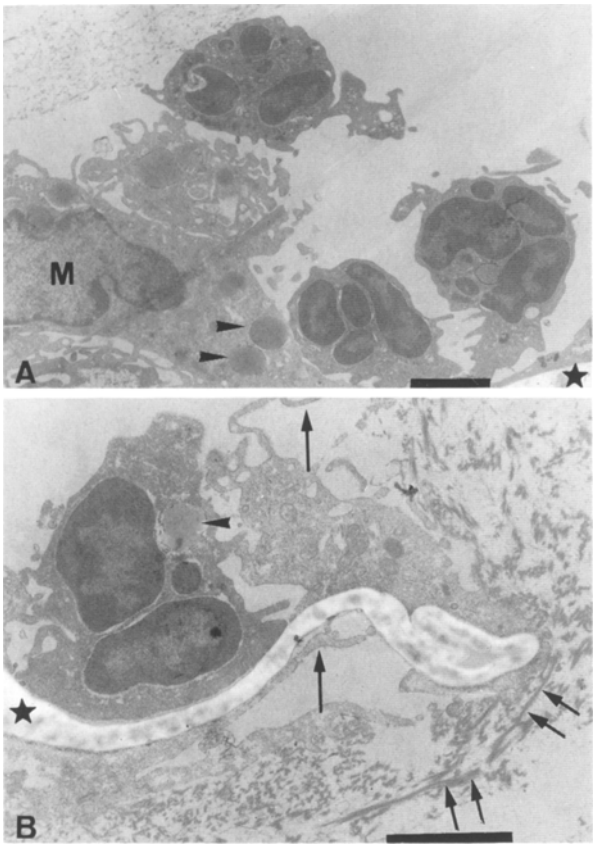

Fig. 5. (**A–B**). TEM. 4 days implanted GCs. (**A**) A macrophage (M) spreading on a plastic mesh (★), presenting lipid vacuoles (*arrowheads*) and surface projections, among neutrophils. × 5000. (**B**) A neutrophil containing a lipid vacuole (*arrowhead*), densly covered by fibrin (*double arrows*) spreading on a plastic mesh (★), together with other cells sectioned so that their nuclei are not apparent, presenting long filopodiae (*arrows*). × 8000. *Bars* = 2 μm

On electron microscopy sponges appeared to be "encrusted" when examined at low magnification by SEM (Fig. 3A). Higher magnification showed that the ECMA covered the plastic meshes as an adherent physiological carpet or forming long fibrillar structures anchored on the pore edges (Fig. 3B) where clambering inflammatory cells or entrapped erythrocytes were observed (Fig. 3C). The cells of the first inflammatory wave migrated actively through the mazy-like sponge pore pathways (Fig. 3D), exhibiting long surface extensions (filopodiae and/or lamellapodia) (Fig. 4A–C) and vacuoles containing lipids (Fig. 5A, B). At 7 days, close to the interface between the two waves, monocytes could be observed expressing long filopodiae in contact with the fibrinolyzed ECMA, together with numerous endothelial cells migrating isolatedly or forming tortuous bloodless or blood-filled very immature capillaries (Fig. 6A). This region examined by SEM showed these capillaries migrating faster than the other elements of the second inflammatory wave, invading the fine fibrillar ECMA and keeping an intimate contact with this provisionally 'adopted' matrix (Fig. 6B, C). Those migrating a little behind were surrounded by the clearly different ECMC (Fig. 7A, B), where few collagen fibrils and new born vessels still presenting signs of immaturity could be observed (Fig. 7C). This granulation tissue, gradually changed its pattern from a loose fibrillar one on the interface between waves to a denser one in the external pores of the sponges, which was even more compact at 14 days (Fig. 8A). The latter, presented an extracellular matrix (ECM) rich in collagen fibrils, where migrating cells frequently showed smoother surfaces (Fig. 8B) than the 'spidery' aspect they exhibited during the first inflammatory wave. Blood vessels generally presented well formed basal membranes, complete endothelial cell junctions and pericytes (Fig. 8C). The filters examined by SEM, showed that the ECMA deposited on the bottom of the chambers until the 7th day, had a delicate and regular fibrillar pattern and during this period of the inflammatory responses seemed to actively be formed and deposited in considerable amounts, covering practically all the migrating inflammatory cells at the bottom of the chamber (Fig. 9A, B). At 14 days, matricial and cellular elements of the second inflammatory wave were already observed on the chamber bottom (Fig. 9C). Fine capillaries (Fig. 9D) as well as tips of ECMC were observed, anchored on the ECMA (Fig. 9E) without the long fibrillar pattern seen on the 4th and the 7th days. These images of the remodelling of the ECMA were achieved due to the retarded overlapping of the first inflammatory wave that the method provided.

Discussion

This is a simple and effective method for fractionation of the inflammatory reaction in vivo. Due to the marked difference between the migratory speed of the acute and the chronic phases throughout the pores of implanted GCs, it was possible to split the inflammatory response into two well separated waves. This experiment showed that the first inflammatory wave had already crossed the whole thickness of the gradient sponges by the 4th day. The second had started its migration on the 4th day, had colonized about one third of the thickness of the sponge at 7 days and had already reached the bottom of the chamber by the 14th day. Longer or shorter intervals between the arrival of the in-

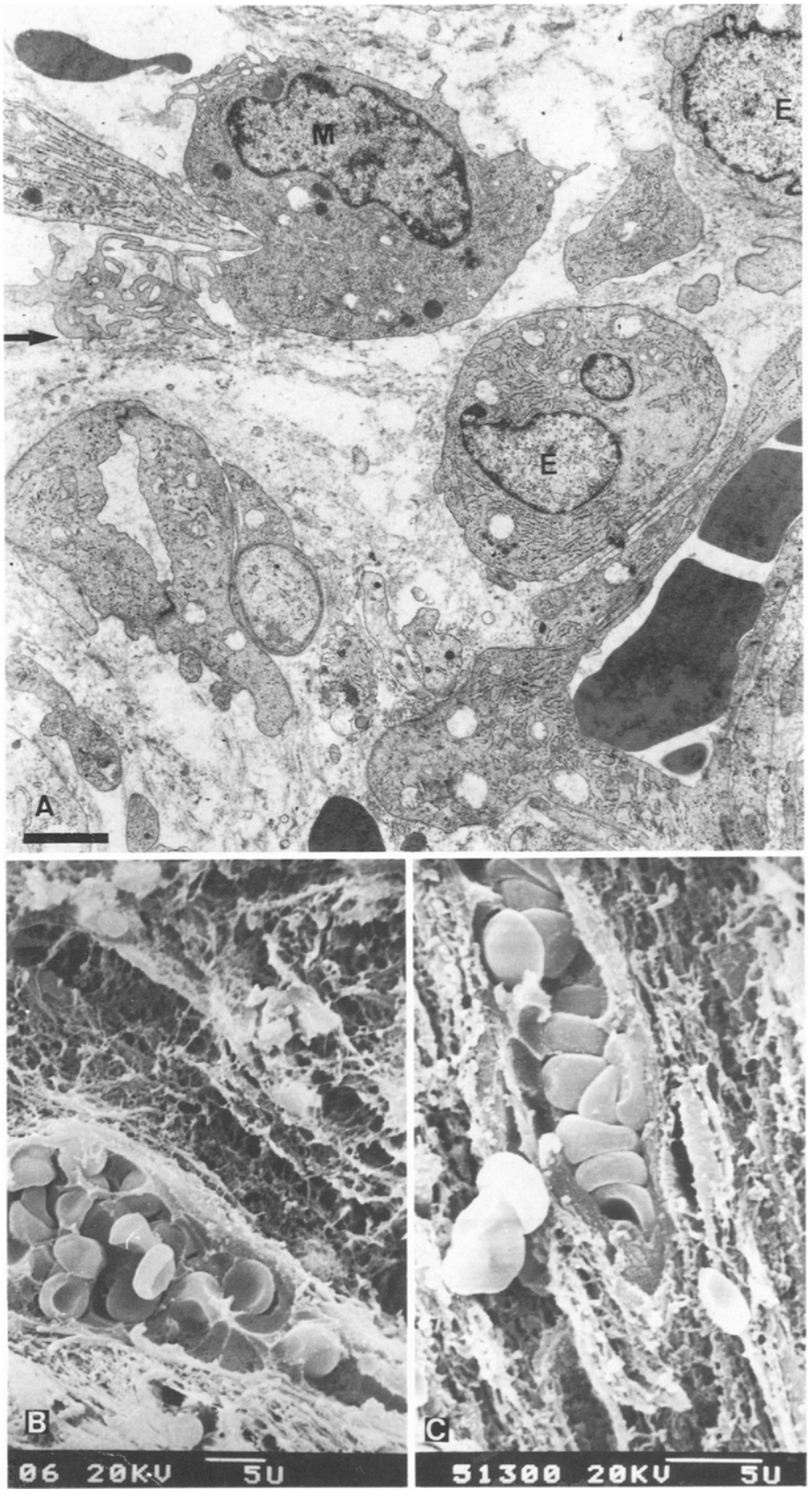

Fig. 6. (**A–C**). Interface between the two waves inside 7 days implanted GCs. (**A**) TEM. Endothelial cells (E) migrating isolatedly or forming very immature bloodless and blood-filled tortuous capillaries. A monocyte (M) already express a well developed filopodia (*arrow*). ×6000. *Bar* = 2 μm. (**B–C**) SEM. Rapidly migrating capillaries invading the ECMA. **B** = ×1800 and **C** = ×3000. *Bars* = 5 μm

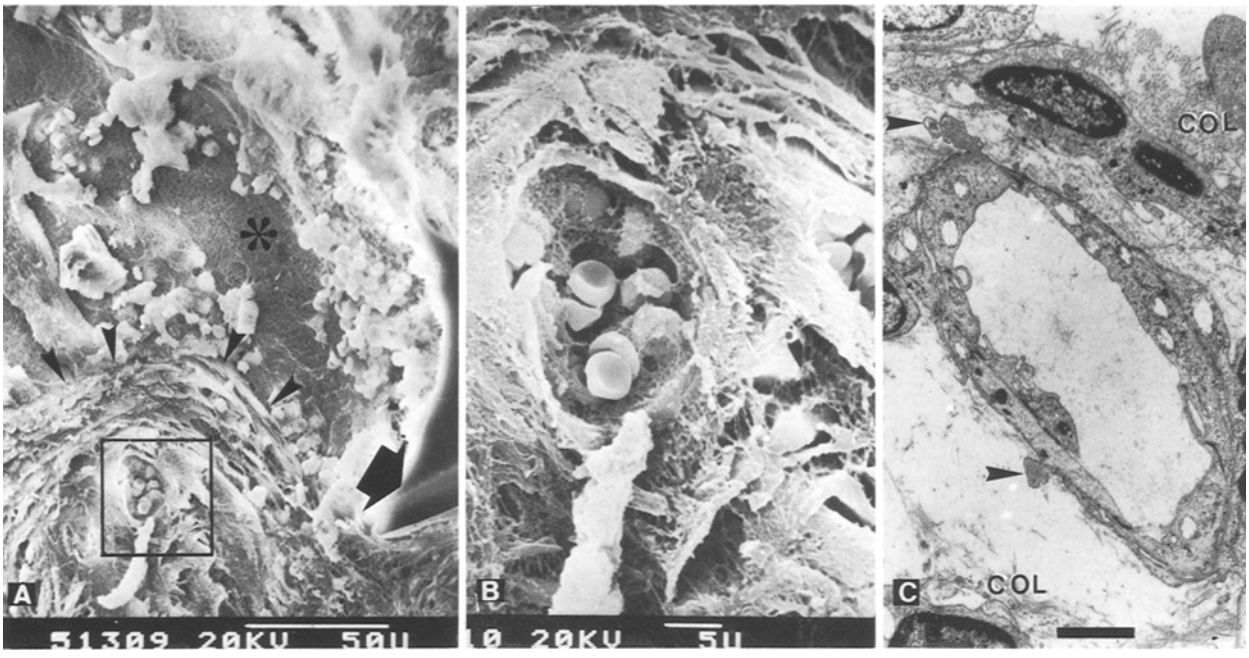

Fig. 7. (**A–C**) 7 days implanted GCs. (**A–B**) SEM. (**A**) The second inflammatory wave (*arrowheads*) overlap the first one (o), crossing a pore where one plastic margin (*arrow*) is cross-sectioned. ×400. *Bar* = 50 μm. (**B**) Magnification of area in **A**. Blood vessels surrounded by denser matrix than the ECMA but still presenting a fibrillar network. ×1800. *Bar* = 5 μm. (**C**) TEM. Front line of the second wave with scarce collagen fibrils (col) and an immature blood vessel lined by activated endothelial cells with cytoplasmic processes projected outwards (*arrowheads*). ×5000. *Bar* = 5 μm

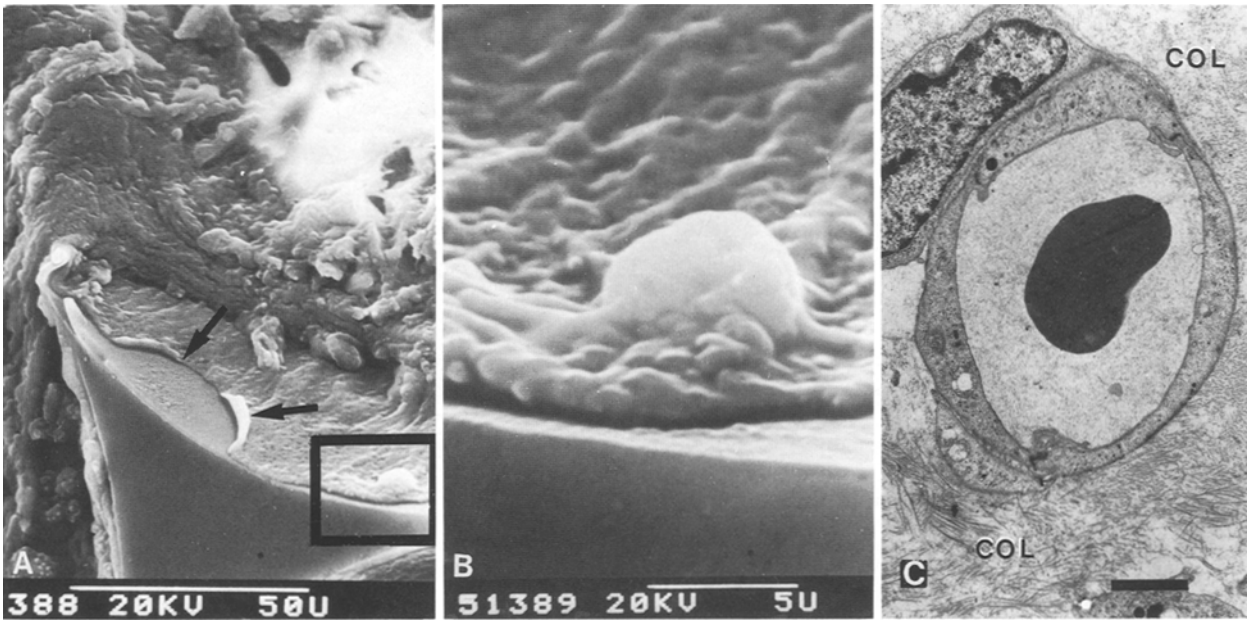

Fig. 8. (**A–C**) Outer pores of GCs at 14 days. (**A–B**) SEM. (**A**) A dense connective tissue adhered to the plastic meshes, retract and fold in (*arrow*) during sponge cross-sectioning. ×660. *Bar* = 50 μm. (**B**) Magnification of area in **A**. The few cells observed spreading under this compact ECMC present a round shape in contrast to those migrating under the ECMA. ×4200. *Bar* = 5 μm. (**C**) TEM. This ECM is rich in collagen fibrils (col), and vessels often present well formed basal membranes, complete endothelial cell junctions and pericytes. ×5000. *Bar* = 2 μm

Fig. 9. (**A–E**) SEM. Inner surface of the filters at the chambers' bottom. (**A–B**) At 7 days, (**A**) filters are already covered by an abundant fibrillar ECM. × 1000. *Bar* = 5 μm. (**B**) Fibrils are long and loosely arranged. × 5800. *Bar* = 5 μm. (**C–E**) The second inflammatory wave on the chambers' bottom at 14 days. (**C**) A large ribbon of viscous-like ECM (o) overlap the ECMA, no more presenting the fibrillar pattern shown at 7 days. × 340. *Bar* = 50 μm. (**D**) Magnification of area in **C**. Capillary (*arrow*) boardered by spindle-shaped cells. × 2000. *Bar* = 5 μm. (**E**) Tips of a granulation tissue anchoring on the ECMA. × 2400. *Bar* = 5 μm

flammatory waves at the chamber bottom might be achieved by increasing or decreasing the thickness of the gradient sponges. As for diffusion chambers, intraperitoneal or subcutaneous implantations of GCs could be performed. The use of sponges made of viscose cellulose (Visella) (Viljanto and Kulonen 1962) and the implantation of a glass coverslip on the chamber bottom for sequential cytological studies (Mesquita et al. 1987) are among other possible technical approaches and/or adaptations, performable with this diffusion-migration chamber.

The widespread assumptions about reciprocal regulatory relationships between ECM components and cells during the biological processes (Hay 1981; Trelstad 1984), emphasizes the importance of analysing cell behaviour in its natural scenario upon its multiple systemic and local mediators. By preventing the rapid overlapping of the elements of the first inflammatory wave by those of the second, the GCs seem to provide a privileged site to study the remodelling process of the ECMA and its interrelation with migrating cells during the acute phase.

Experiments done with diffusion chambers have indicated that Millipore chambers provide a suitable milieu for living transplants (for general references see Carsten 1984). In the diffusion-migration chamber described in this paper, preliminary results obtained with *Paracoccidioides brasiliensis* implanted inside GCs in rats (Mesquita 1987b) as well as some other targets already tested (sarcoma 180 implanted in mice, unpublished data), seem to indicate that this favourable outcome is not altered by the inclusion of a sponge in the system. They support the value of setting implants inside GCs. However, all systemic conditions capable of affecting the host's inflammatory response as well as local specific factors released by target implants may change the migratory speed and tissue pattern described inside the empty GCs.

Despite the large number of microscopic studies already performed, our data represents the first ultrastructural examination, by TEM and SEM, of implanted plastic sponges. For a better evaluation of the potential of this bioassay to study the cell-matrix and cell-to-cell interaction and the turnover and behaviour of these elements during inflammatory reactions, more experiments with GCs using modern techniques will be necessary. It is conceivable that the possibility of splitting the inflammatory response into two well separated waves and analysing sequentially the interrelation between these waves and the target implants presents a new approach that may allow a novel contribution to the study of the inflammatory process in vivo.

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