

Morphological and Biochemical Characterization of Macrophages Activated by Carrageenan and Lipopolysaccharide *In Vivo*

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ABSTRACT. Macrophages are able to recognize, internalize and destroy a large number of pathogens, thus restricting the infection until adaptive immunity is initiated. In this work our aim was to analyze the surface charge of cells activated by carrageenan (CAR) and lipopolysaccharide (LPS) through light and electron microscopy approaches as well as the release of inflammatory mediators *in vitro*. The ultrastructural analysis and the light microscopy data showed that *in vivo* administration of CAR represents a potent inflammatory stimulation for macrophages leading to a high degree of spreading, an increase in their size, in the number of the intracellular vacuoles and membrane projections as compared to the macrophages collected from untreated animals as well as mice submitted to LPS. Our data demonstrated that CAR stimulated-macrophages displayed a remarkable increase in nitric oxide production and PGE2 release as compared to the cells collected from non-stimulated and stimulated mice with LPS *in vivo*. On the other hand, non-stimulated macrophages as well as macrophages stimulated by LPS produce almost the same quantities of TNF- α , while *in vivo* stimulation by CAR leads to a 30–40% increase of cytokine release *in vitro* compared to the other groups.

In conclusion, our morphological and biochemical data clearly showed that *in vivo* stimulation with CAR induces a potent inflammatory response in macrophages representing an interesting model to analyze inflammatory responses.

Key words: macrophages/carrageenan/lipopolysaccharide/electron microscopy

Introduction

Macrophages can be divided into normal and inflammatory macrophages. The former includes macrophages in connective tissue (histiocytes), liver (Kupffer cells), lung (alveolar macrophages), lymph nodes, spleen, bone marrow, skin (histiocytes, Langerhans cells) and others. Inflammatory macrophages are characterized by their various specific markers and share similar properties to the monocytes (Rosenberger and Finlay, 2003; Maurer *et al.*, 2002).

Macrophages play a fundamental role in both humoral

and cellular immune responses. They are able to rapidly recognize, internalize and destroy a large number of pathogens, thus restricting the infection until the cell host is able to initiate adaptive immunity (reviewed in Rosenberger and Finlay, 2003). They present antigens to lymphocytes, modulate T cell functions, and secrete a large number of inflammatory mediators, which play roles in the amplification of both humoral and cell-mediated immune responses. (Jedynak and Siemiatkowski, 2002).

Due to their varied roles and the environmental stimuli that they receive, macrophages exhibit different phenotypes, which are mostly related to their morphology, cell surface antigen expression and function. This phenotypic heterogeneity is a consequence of a series of down-regulations of certain cellular processes and the up-regulation of others. (Arandjelovic *et al.*, 1998). The appearance and activation of macrophages are thought to be rapid events in the development of many pathological lesions, including malignant

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Abbreviations: LPS, Lipopolysaccharide; CAR, Carrageenan; CF, Cationized Ferritin.

tumors, atherosclerotic plaques, and arthritic joints. Recently, macrophages have been used as novel cellular vehicles for gene therapy (Burger and Dayer, 2002; Greaves and Gordon, 2002).

In the literature, several agents have been used to promote macrophage activation, including the lipopolysaccharide (LPS) and the carrageenan (CAR) molecules (Risco and Pinto da Silva, 1995; Nacife *et al.*, 2000; Tsuji *et al.*, 2003). LPS is a bacterial endotoxin, which acts on numerous cellular functions through the processes of cell activation and damage. The molecular mechanisms involved in the “endotoxic phenomenon” are still not completely understood (Risco and Pinto da Silva, 1995).

Carrageenan is known to induce acute inflammatory exudation (Winter *et al.*, 1962) and paw edema (Vinegar *et al.*, 1969; Henriques *et al.*, 1987), injury to blood vessels (Ward and Cochrane, 1965), inhibition of complement functions (Davies, 1965) and kinin release (Di Rosa and Sorrentino, 1970). It is also used to induce the formation of air pouch in rodents (Sedgwick and Lees, 1986), followed by cell accumulation and mediator release (Romano *et al.*, 1997).

Up to now, few data are available concerning the morphological and biochemical aspects related to the cells activated by CAR, hence our present aim is to characterize some properties of mice macrophages stimulated by CAR *in vivo* through light and electron microscopy assays. Besides, we also analyzed the release of some inflammatory mediators *in vitro* comparing to the data obtained from another well-known inflammatory stimuli, LPS.

Material and Methods

Materials

Carrageenan λ type IV and LPS (from *Escherichia coli*) were purchased from Sigma Chemical Co. USA.

In vivo stimulation

Swiss male mice (18–20 g) were intraperitoneally injected with 300 μ g CAR or 250 ng LPS in saline as described (Nacife *et al.*, 2000). In the control group, mice did not receive any type of stimulation.

Peritoneal cell cultures

Peritoneal cells from the different groups of mice were collected using Dulbecco’s modified Eagle medium (DME) without serum. For light microscopy analysis and inflammatory mediators measurements, after the determination of the cell density by Neubauer chamber quantification, the obtained cells were plated into 24-well microplates at a cell density of 10^6 cells/well, with 10% of fetal calf serum (DMES) or with supplementation of 2% serum bovine albumin for light and inflammatory mediators measurements, respectively.

Light microscopy

After 24 and 48 hours of cells plating, untreated and CAR- and LPS-treated peritoneal cells, were fixed in Bouin’s solution, stained with May-Grünwald-Giemsa and analysed at light microscopy level.

Transmission electron microscopy (TEM)

Peritoneal cells from untreated mice and after stimulation for 24 and 48 hours with CAR and LPS were collected, fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and 3.5% sucrose for 1 hour at 4°C, post-fixed with 1% osmium tetroxide in the same buffer for another hour at 4°C. They were dehydrated in a graded series of acetone and embedded in Epon resin. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with Zeiss electron microscope (EM 10C).

Surface charge analysis

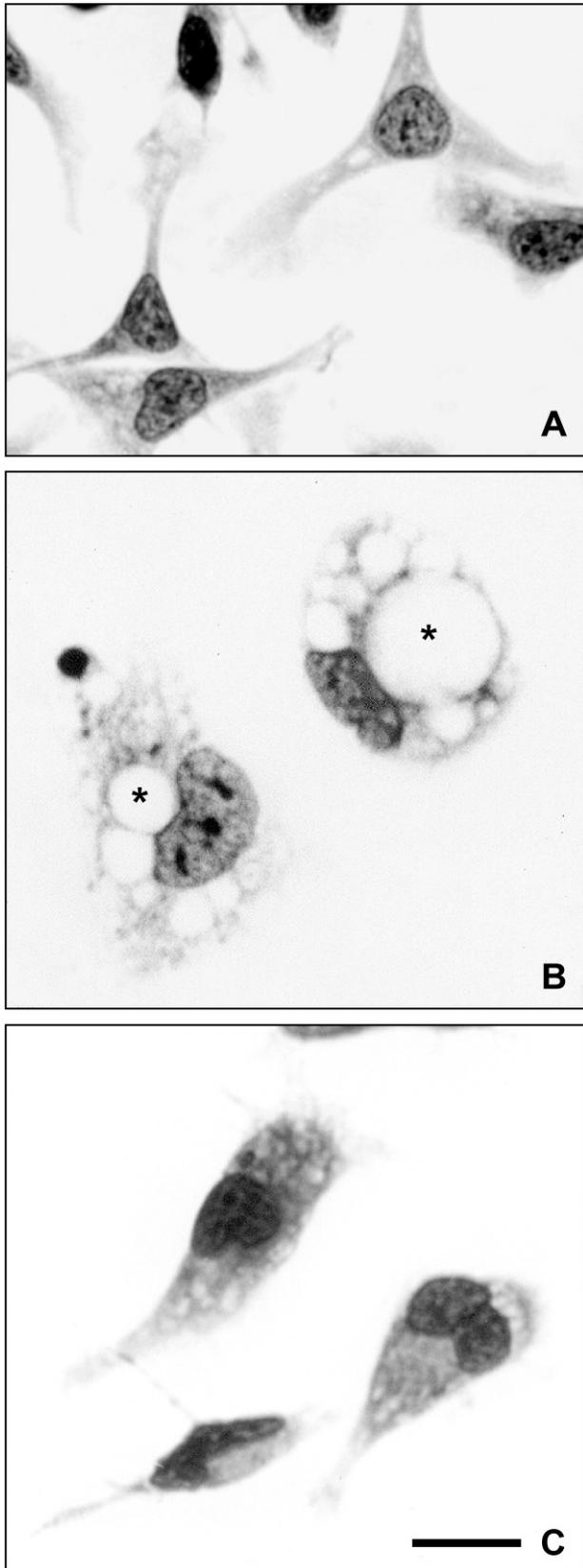
Peritoneal macrophages from untreated mice and after 24–48 hours of *in vivo* stimulation with CAR and LPS were collected using Dulbecco’s modified Eagle medium. The cells were then washed in cold saline and immediately treated for 30 minutes at 4°C with 100 μ g·ml⁻¹ cationized ferritin (CF) and processed for TEM as described above.

Inflammatory mediators measurements

After 24 and 48 hours of *in vivo* stimulation by CAR and LPS, the peritoneal cells were collected as described and seeded in 24-well microplates at a cell density of 10^6 cells/well. After 24 and 48 hours of culture plating (grown in the absence of serum sources but supplemented with 1% albumin serum bovine), the supernatants were collected and stored at –70°C for analysis of nitric oxide (NO), tumoral necrosis alpha factor (TNF- α) and prostaglandins E2 (PGE2) levels, and fresh medium was added to the microplates. The quantification of NO was done by mixing 50 μ l of the culture supernatant with an equal volume of the Griess reagent in a 96-well flat-bottomed microtiter plate. This reagent was prepared by mixing equal volumes of 1% sulphanilamide in water containing 5% phosphoric acid with 0.1% N-(1-naphtyl) ethylenediamine hydrochloride (Ding *et al.*, 1988). After 15 min at room temperature, the plates were read at 540 nm. In each individual plate, duplicates of a sodium nitrite solution in eight concentrations from 0 to 100 μ M were used to generate standard curves. For TNF- α and PGE2 analysis we performed an ELISA assay according to the manufacturer’s instructions (Duo Set Kit, R&D, Cayman Chemical, Ann Arbor, MI).

Results

At light microscopy level, cells obtained from the peritoneum of CAR-injected mice showed a higher degree of spreading, increased number of membrane projections and a large number of vacuole profiles (Fig. 1B) as compared to both untreated (Fig. 1A) and LPS-treated (Fig. 1C) mice.



The analysis by TEM of the CAR and LPS-stimulated macrophages showed similar results after 24–48 hours of the *in vivo* treatment and confirmed the light microscopy results. The peritoneal cells collected after CAR stimulation displayed a higher number of membrane projections and cytoplasmic vacuoles (Fig. 2B and 2C) as compared to the non-stimulated macrophages (Fig. 2A). However, the analysis of the LPS-stimulated cells showed a greater number of cytoplasmic projections (Fig. 2D) as compared to the control cells (Fig. 2A) but a smaller number of vacuoles as compared to the CAR-treated mice group (Fig. 2B and 2C).

Since the surface properties of the membranes from the CAR activated-macrophages are not well understood and may be involved in the altered morphology observed both by optical and electron microscopy analysis, we next investigated their surface charge by means of cationized ferritin (CF) binding. After incubation for 30 min at 4°C in the presence of CF, the untreated macrophages were intensively labeled all over their surface (Fig. 3A). However, stimulation by CAR *in vivo* resulted in a drastic decrease in the binding capacity of the cell membrane to the cationized ferritin particles suggesting a modulatory effect on the surface charge of macrophage plasma membrane by CAR (Fig. 3B). On the contrary, the samples collected after LPS stimulation did not present significant differences (Fig. 3C) concerning the amount and distribution of the positive tracer towards the anionic surface sites as compared to the untreated control (Fig. 3A).

Next, we investigated the release of certain inflammatory mediators at the supernatant of the macrophages collected after induction or not by CAR and LPS (Table I). Our results show that peritoneal cells obtained from non-stimulated mice released increasing amounts of nitrite along with the length of time of their *in vitro* culture: about 13.7 and 29.9 μM , after 24 and 48 hours of plating, respectively. Stimulated-CAR macrophages displayed higher levels as compared to the control cells reaching 33.2 and 52.6 μM and displaying rises of 142 and 75% after 24 and 48 h plating, respectively. On the other hand, LPS group displayed less increase as compared to CAR treated mice reaching about 22.1 and 39.4 μM of nitrite release in the culture supernatant, displaying 61 and 31% of increase as compared to untreated samples (Table I).

The measurement of PGE2 release in the supernatant from non-stimulated and stimulated macrophages by CAR and LPS after 24 hours of culture plating showed levels of 8.8, 15.2 and 9.6 ng/ml, respectively; which proportionally increased slightly in all groups after 48 hours of plating,

Fig. 1. Light microscopy of mice peritoneal cells in Giemsa-stained slides showing the characteristic morphology of non-stimulated (A), CAR-stimulated (B) and LPS-stimulated macrophages for 48 hours *in vivo*. Note the increase of spreading and cytoplasmic vacuole profiles (asterisk) in the CAR-stimulated macrophages (B) as compared to the non-stimulated (A) and LPS treated mice (C). Bar=25 μm

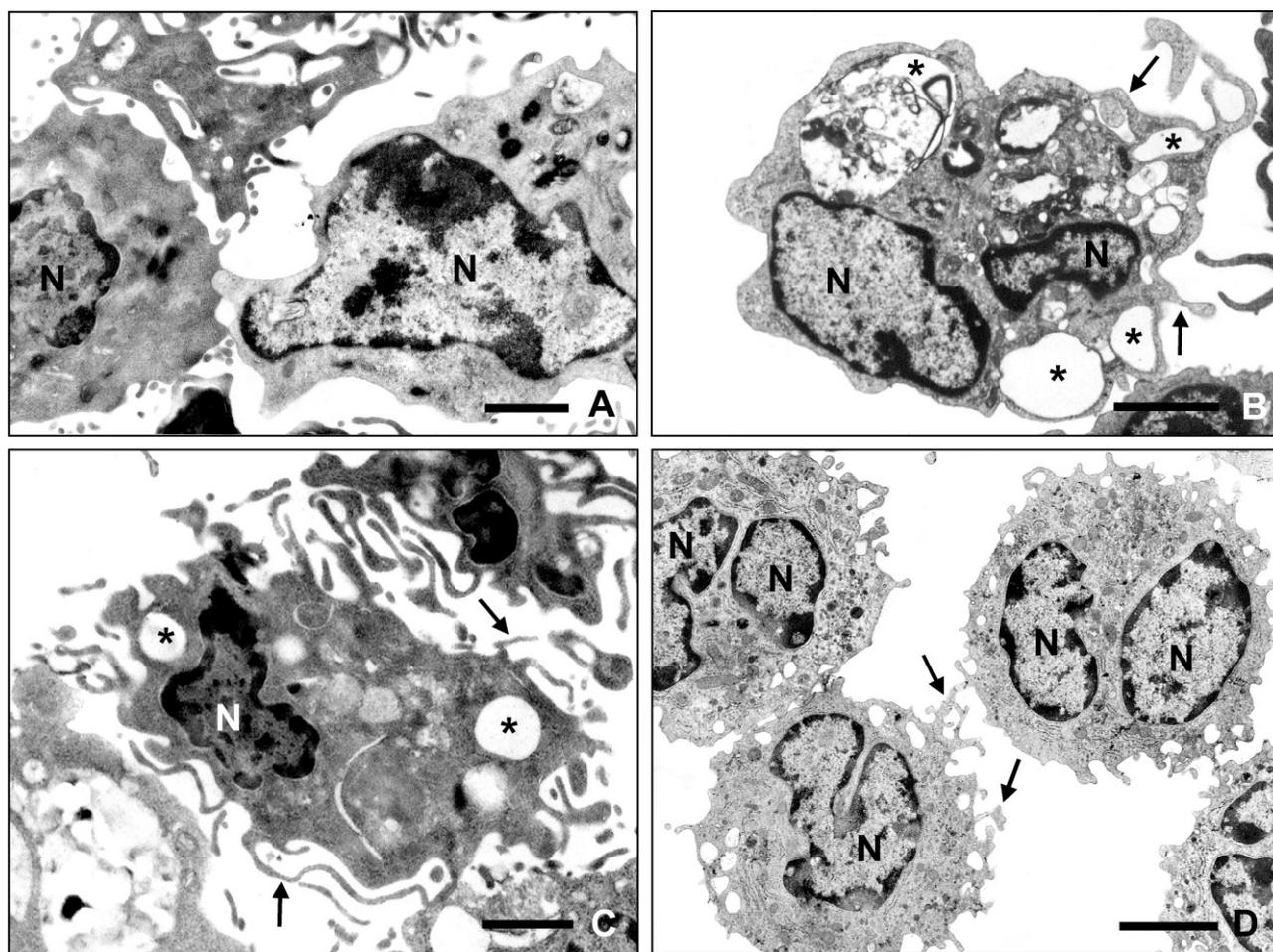


Fig. 2. Ultrastructural aspects of mice peritoneal cells showing the characteristic morphology of non-stimulated (A) LPS-stimulated (D) and CAR-stimulated macrophages for 24 (B) and 48 hours *in vivo* (C). Note the huge increase in the number of intracellular vacuoles (asterisk) and membrane projections (arrow) in the CAR-stimulated macrophages (B and C) as compared to the non-stimulated (A) and LPS treated mice (D). Bar=1 μ m

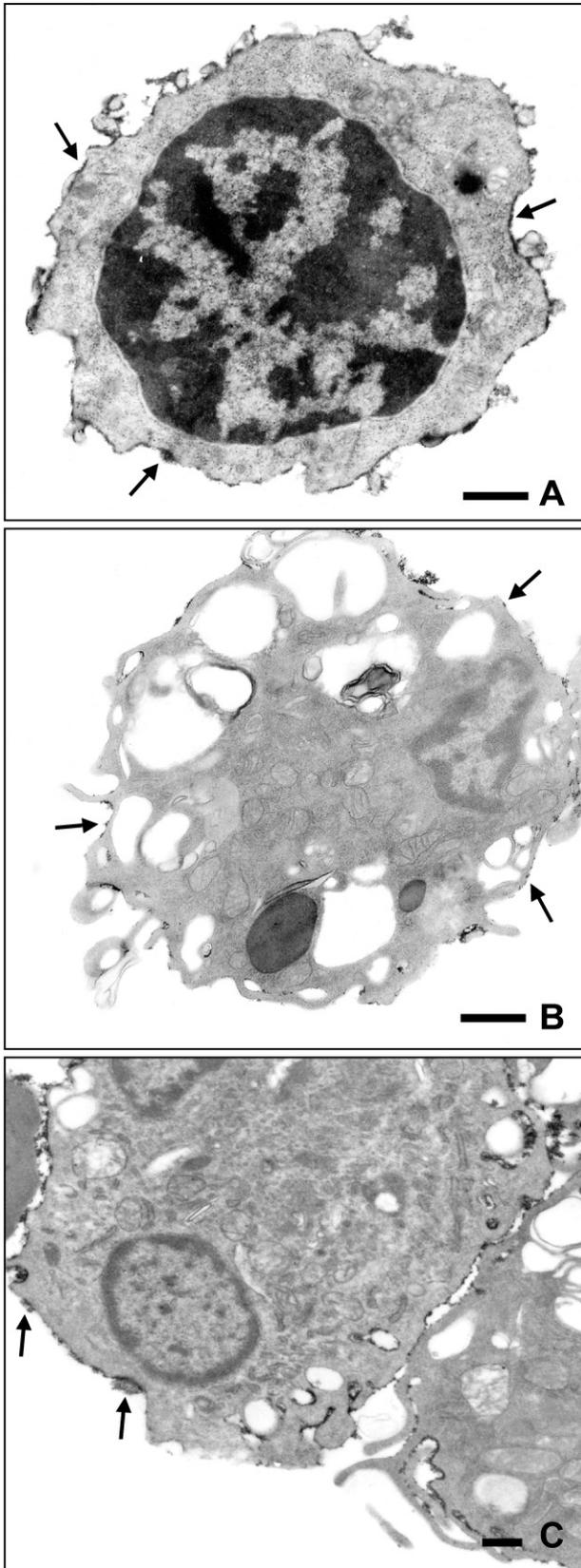
reaching 10.5, 17.0 and 12.8 ng/ml (Table I). As noted with nitrite production, the CAR stimulated group displayed higher levels of PGE₂ release as compared to untreated and LPS treated mice, reaching 72 and 61% of mediator release after 24 and 48 hours of plating as compared to the cells obtained from non-stimulated mice. On the other, comparing to non-stimulated group, LPS treatment led to a small rise of PGE₂ release in the supernatant, displaying increases of 9 and 22% after 24 and 48 hours of cell culture, respectively.

The measurement of another inflammatory mediator, TNF- α , showed that only the CAR stimulated group presented a small increase in cytokine release (29% and 43% of increase) in the culture supernatant as compared to the non-stimulated macrophages, after 24 and 48 hours of plating, respectively. LPS always showed similar values as compared to cells collected from non-stimulated mice (Table I).

Discussion

Macrophages function as both sentinels and the first line of defense against infection, and therefore are an essential barrier that pathogens must overcome to be successful. They have a fundamental role in humoral and cellular immune responses being involved in a variety of immunological and inflammatory processes from antigen presentation to microbicidal and tumoricidal activities (reviewed in Gordon, 1998; Hubbard, 1999).

Our present results showed that *in vivo* administration of CAR represents a potent inflammatory stimulation for peritoneal macrophages leading to a higher degree of spreading, an increase in their size, increase in the number of membrane projections and an overall enhancement in the number and size of intracellular vacuoles as compared to the cells obtained from untreated and even submitted to another inflammatory stimuli such as LPS. The ultrastructural analy-



sis confirmed the light microscopy data and clearly showed that although these cells displayed altered morphology mostly due to the presence of large vacuoles and membrane projections, they were not damaged. They presented plasma membrane integrity and well-preserved mitochondria profiles characteristic of good cell viability. Our results showed that peritoneal cells collected from the LPS-treated group also displayed altered morphology, however, it was less intense as compared to the CAR stimulation. Our present data confirmed previous report, which showed that CAR injection did not affect cell viability measured by the incorporation of propidium iodide (Nacife *et al.*, 2000). The authors showed that the increased number of vacuoles during CAR stimulation was probably due to the increased exocytosis of inflammatory proteins, which could be detected in their culture supernatant. In fact, our present results concerning the measurements of the inflammatory mediators also showed that CAR induced a higher degree of inflammatory release as compared to that of mice injected with LPS.

Macrophages like others vertebrate cells have a negative charge due to the membrane anionic components (Weiss, 1969; Burry and Wood, 1979; Meirelles *et al.*, 1984; Mutsaers and Papadimitriou, 1988). The surface charge plays an important role in cell-cell interactions, cellular differentiation, adhesion and endocytosis (van Oss, 1978; Papadimitriou, 1982; Spangenberg and Crawford, 1987; de Carvalho and de Souza, 1990). In the present work, we observed that macrophages collected from mice stimulated by CAR showed a decrease in the number of CF particles in their surface as compared to the macrophages obtained from non-stimulated and LPS-stimulated mice. Alterations of the surface charge also have been described in erythrocytes, which paralleled changes in their morphology (Samoilov *et al.*, 2002). On the other hand, macrophages elicited by thioglycolate did not display any differences concerning the binding of cationized ferritin particles at pH 7.2 to the cell surface as compared to resident and macrophages (Santos and De Souza, 1983). Since the macrophages have an important endocytic and exocytic activities, which involves the cell surface properties (Silva Filho and De Souza, 1988; Mutsaers and Papadimitriou, 1988; Rabinowitz and Gordon, 1989), the reduced anionogenicity presently observed in the CAR-activated macrophages could lead to alterations in the endocytic/exocytic pathways of these cells, which has been previously suggested during internalization assays employing fluorescent probes such as lysotracker yellow

Fig. 3. Electron micrograph of macrophages after incubation with cationized ferritin. A remarkable reduction in the number of cationized ferritin particles (arrow) associated to the surface of CAR-stimulated macrophages (B) as compared to untreated samples (A) is evident. Conversely, no great differences were observed in the amount and distribution of the positive tracer towards the anionic surface sites (arrow) in the LPS-treated group (C), as compared to the untreated control (A). N=Nucleus. Bar=0.5 μ m

Table I. MEASUREMENTS OF NO, PGE2 AND TNF- α IN THE SUPERNATANTS COLLECTED FROM NON-STIMULATED, LPS- AND CAR-STIMULATED CULTURES OF MICE PERITONEAL MACROPHAGES *in vivo*

| Hours after culture plating | NO (μ M) | | PGE2 (ng/ml) | | TNF- α (ng/ml) | |
|------------------------------------|----------------|----------------|----------------|----------------|-----------------------|------------------|
| | 24 | 48 | 24 | 48 | 24 | 48 |
| Non-stimulated macrophages | 13.7 \pm 1.5 | 29.9 \pm 1.4 | 8.8 \pm 1.2 | 10.5 \pm 1.3 | 0.073 \pm 0.06 | 0.133 \pm 0.04 |
| Carrageenan-stimulated macrophages | 33.2 \pm 3.9 | 52.6 \pm 3.6 | 15.2 \pm 3.4 | 17.0 \pm 3.4 | 0.094 \pm 0.1 | 0.191 \pm 0.1 |
| LPS-stimulated macrophages | 22.1 \pm 2.8 | 39.4 \pm 2.6 | 9.6 \pm 2.2 | 12.8 \pm 2.3 | 0.073 \pm 0.04 | 0.13 \pm 0.06 |

and acridine orange (Nacife *et al.*, 2000). Further studies are now under way to further investigate these pathways in CAR stimulated cells using specific ligands for the receptor mediated- and fluid phase endocytosis.

At the site of an inflammatory reaction, injured vascular endothelial cells and emigrated leukocytes release several inflammatory mediators, which modulate and maintain the inflammation. Carrageenan is used as a component to induce non-specific inflammation, possibly through activation of innate immune responses. It modulates acquired immunity, adjuvant and suppressive effects (Coste *et al.*, 1989; Macino and Morelli, 1983; Bash and Vago, 1980; Nicklin and Miller, 1984; Cochran and Baxter, 1984; Vijayakumar *et al.*, 1990). In our results, we observed that CAR stimulated-macrophages displayed a remarkable increase (142–75%) of nitric oxide production as compared to non-stimulated cells and a significant increase of about 50–33% as compared to the macrophages collected from mice stimulated with LPS. The role of NO in the inflammation has been deeply investigated. NO is generated from L-arginine by a family of enzymes called NO synthases (NOS) (Morris and Billiar, 1994). NO has been shown to have pro- and anti-inflammatory actions (Darley-Usmar *et al.*, 1995). It has been reported that when NO is generated by the constitutive isoform of NOS in the vascular endothelium, this messenger inhibits both neutrophil adhesion to the endothelium (McCall *et al.*, 1988) and to the postcapillary venules (Kubes *et al.*, 1991; Arndt *et al.*, 1993). Regarding carrageenan, it is known that NO increases plasma extravasation, paw edema (Ialenti *et al.*, 1993) and granuloma formation (Iuvone *et al.*, 1994) induced by CAR stimuli. In another study, it has been reported that the intraplantar injection of this agent increases the levels of NO in the model of paw edema, which can be inhibited by NO inhibitors, such as NG-monomethyl-L-arginine and NG-nitro-L-arginine methyl ester (Salvemini *et al.*, 1996).

Upon stimulation by interferon- γ (IFN- γ), tumor necrosis factor α (TNF- α) and lipopolysaccharide (LPS), macrophages express a Ca²⁺/calmodulin-independent NOS, leading to NO production (Nathan, 1992; Jorens *et al.*, 1995). The

NO produced by macrophages mediates the cytostatic and cytotoxic effects to a variety of pathogens including viruses, bacteria, protozoa and helminthes (Sternberg and McGuigan, 1992; Mabbott *et al.*, 1994; Visser *et al.*, 1995; revised by James, 1995). In our present study, we showed that the effects of CAR on peritoneal cells reflect its high ability as an inflammatory stimulus, being superior to the effects of LPS.

Besides the analysis of NO release, we also assayed the PGE2 production by peritoneal cells collected from CAR-stimulated mice. Prostaglandins are well-known inflammatory mediators (Hunter, 2002; Kast, 2001, Kuraishi and Ushikubi, 2001) and the literature data showed that macrophages stimulated by CAR can produce PGE2, which could be responsible for the immunosuppressive activities of CAR, which is related to the dosage, route of administration and fraction used to perform the experiments (Bash and Vago, 1980). In our present analysis we found a striking increase of PGE2 release in the supernatant of cultured peritoneal cells after *in vivo* stimulation as compared to non-stimulated and LPS-treated mice (70 and 58% of increase, respectively).

In inflammatory reactions induced by λ -CAR, the role of TNF- α has already been investigated. In models of carrageenan-induced pleurisy it has been noted that TNF- α levels were significantly elevated in the exudates (Cuzzocrea *et al.*, 1999). In our results, we observed that non-stimulated macrophages as well as macrophages stimulated by LPS produce almost the same quantities of TNF- α ; however after *in vivo* stimulation by CAR we found a slight increase of 30–40% in the cytokine release *in vitro* as compared to the other groups.

In summary, we observed that cells stimulated with carrageenan showed a number of characteristics of activated cells, such as altered morphology due to a greater number of membrane projections and a greater number of vacuoles as compared to non-stimulated macrophages as well as to macrophages from mice stimulated *in vivo* with LPS. The biochemical (high release of inflammatory mediators) data in the present report also argue for the role of CAR as a potent

inflammatory agent and recruiter of monocytes and macrophages. Further biochemical studies are presently under way to better clarify this activation induced by carrageenan.

Acknowledgments. We would like to thank Bruno Ávila for help with image processing. This investigation was supported by FAPERJ, CNPq and Fundação Oswaldo Cruz, Instituto Oswaldo Cruz.

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(Received for publication, January 20, 2004

and accepted, April 15, 2004)