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In Vitro Stimulation of Murine Peritoneal Monocytes Induced by Alginates

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In this trial we assessed the effect of soluble alginates on murine cells. Mouse peritoneal monocytes were stimulated *in vitro* with a solution of alginate. The production of TNF-α and nitric oxide (NO), the expression of surface molecules CD80 and CD86, and the ability of monocytes to phagocyte bacteria were assessed, in order to evaluate the effect of alginate on cell functionality. We showed that mouse peritoneal monocytes stimulated with alginate produce NO and TNF-α. In addition, alginate is able also to increase their phagocytic activity and to a lesser extent also to increase the expression of CD80. Even with different degrees, it implies that alginates *per se* act directly on immune response, being able to effectively stimulate proinflammatory activity. These findings corroborate the idea that alginates can represent interesting adjuvants to use to increase the efficacy of antigenic stimulation.

Key words: Monocytes, Alginates, Immuno-stimulation, TNF-α

INTRODUCTION

The role of vaccines in food animal production is one of increasing importance for several reasons. The impetus to keep production costs down is significant; this is reflected in prevention of disease in lieu of treatment (André, 2001). Consumers are also making greater demands for less antibiotic usage in production for fear of developing microbial resistance (Tauxe, 1998). As vaccination strategies become paramount in management, efficacy of the product is critical and is reflected in several parameters of the product. The type of antigen used greatly affects the duration of immunity.

The route of administration offers insight into the nature of the immune response. Vehicles used to present the antigen are a growing area of investigation. The cumulative effect of these factors comprises the greatest part of vaccine efficacy. The type of antigen utilized in a vaccine to stimulate a immunity similar to natural infection (Ellis, 2001), is often linked to humoral and cellular immunity. Live vaccines include the potential to cause disease by reversion. In contrast, inactivated vaccines require multiple administrations to provide long-term immunity and typically are limited to humoral stimulation. The use of adjuvants, or vehicles, has been employed to overcome these problems, increasing vaccine efficacy (Hess *et al.*, 2000). The composition of the vaccine, excepting the antigen, serves a multifaceted role. It stabilizes the antigen, lengthens antigen presentation and, finally, amplifies the immune response to the antigen through its own reactivity. Adjuvants can increase or facilitate the immune response in order to achieve more effective protection (Ellis, 2001). The most used adjuvants are those based on alum salts; these are safe but they are not always able to induce a sufficient immune-based protection. Immunostimulatory complexes (ISCOMS) are small particles of cholesterol, phospholipids with immunomodulatory effects; their associated immune response is considered ten fold greater than traditional alum/oil adjuvants (Bowerestock and Martin, 1999). Microparticles represent one of the newest categories, especially those based on polysaccharides. Alginates are naturally occurring polysaccharides derived from brown seaweeds. They are block copolymers of homopolymeric sequences of (1→4)-linked β-D-mannuronate (M) and α-L-guluronate (G) residues, linked

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together with blocks of mixed sequences. The proportions and lengths of these blocks, widely depending on the algal source, determine the physical properties both of the biopolymer and of the ensuing gels (Smidsrød and Draget, 1998). When divalent cations as Ca^{2+}, Ba^{2+}, Sr^{2+} are present, alginates might be able to form gels and eventually gel microparticles, if the alginate solution is dripped into a gelling bath. Information gathered about this subset of microparticles suggests great potential in the future of mucosal vaccines. Conditions to induce encapsulation of various antigens are mild and their use has been observed to activate macrophages and to stimulate cytokine production (Bowerestock and Martin, 1999). However, further studies on the role of alginates as adjuvants are needed prior to expanding their application to host and antigen. This trial was performed in order to evaluate the effect of soluble alginates on peritoneal monocytes. In particular, the ability of peritoneal monocytes to produce cytokines and effector molecules was studied after stimulation with alginates, in an attempt to investigate the mechanisms which pave the way to the efficacy of alginates used as adjuvant.

MATERIALS AND METHODS

Animals

BALB/c female mice were obtained from Charles River, Milan, Italy. They were maintained in barrier housing with filtered inflow air in a restricted-access room and in pathogen-limited conditions. They were fed a commercial diet and water was provided *ad libitum*.

Cell culture

At any set of the experiments, resident peritoneal monocytes were collected from 5 BALB/c mice by lavage with 10 mL of RPMI 1640 medium containing 2 mM L-glutamine, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 10% fetal bovine serum, 5×10-M 2-mercaptoethanol, 100 U of penicillin, and 100 μg of streptomycin per mL (RPMI). Cells were centrifuged (800 g ×5 min) and red blood cells were removed by a lysis buffer (NH_4Cl 150 mM, $KHCO_3$ 10 mM, EDTA 0.1 mM). Peritoneal monocytes were then resuspended in RPMI and kept at 37°C and 5% CO_2. For flow cytometry, adherent peritoneal cells were collected and used.

Alginate solutions

A solution of 5% (w/v) of alginate sodium salt from *Laminaria hyperborea* (LH), purchased from Fluka (Milan, Italy), was prepared in sterile saline. Different concentrations of alginate were used according to experimental protocols.

In vitro stimulation

Flat-bottom 96-well plates were used throughout the experiments. Three or 4 replicate cultures were set up for every stimulation throughout the experiment. Peritoneal monocytes (1×10^5) were incubated with different concentration of alginates in a total volume of 200 μL, for different time according to the experimental design. Supernates were collected at different time points to evaluate the production of nitric oxide (NO) and TNF-α. NO production was assessed according to the procedures of Griess Reagent System Cells (Promega, Milan, Italy). In brief, an aliquot of a sulfanilamide solution was add to each well. After 10 minutes another aliquot of *N*-1-napthylethylenediamine dihydrochloride solution was addedd (NED) and the reaction was read at 550 nm with a plate reader. TNF-α production was detected by enzyme-linked immunosorbent assay according to the manufacturer's instruction (R&D Systems, Minneapolis, MN, U.S.A.). To assess the residual toxicity of alginates, cells were monitored to assess their viability according to the procedures of the CellTiter 96 Aqueous One Solution cell proliferation assay kit (Promega, Milan, Italy). Briefly, assays were performed by adding a solution of tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; MTS] directly to culture wells, incubating for 1-4 h and then recording absorbance at 490 nm with a 96-well plate reader. The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture.

Flow cytometry

All steps for flow cytometric analysis were performed on ice. Mouse peritoneal monocytes (2×10^6/mL) were stimulated with different concentration of alginates, for 24 h. After stimulation, cells (2×10^5) were washed twice with fluorescent buffer (phosphate-buffered saline, containing 0.01% sodium azide and 0.1% bovine serum albumin), and incubated with FITC-labeled antibody anti-mouse CD80 and CD86, according to manufacturer's suggestions (Serotec Ltd, Oxford, U.K.). After a 30 minutes incubation on ice, cells were washed twice with fluorescent buffer and then dispersed in 0.5 mL of cold fixative buffer (phosphate-buffered saline, containing 0.01% sodium azide and 1% formaldehyde). Analysis was performed using a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, U.S.A.).

Phagocytosis assay

A modification of the Vybrant Phagocytosis Assay kit (Molecular Probes) was used. In particular, peritoneal monocytes were stimulated with different concentration of alginates. After 24 h, labelled bacteria (Molecular Probes)

were add to cell culture and phagocytosis was allowed for 2 h. Unphagocytosed bacteria were extensively washed out and cells were then analysed to flow citometry. Cell fluorescence was correlated to phagocytosis.

Statistical analysis

Significance of differences between groups was estimated by a one-way analysis of variance. Differences were considered significant when $P \leq 0.05$.

RESULTS

Viability of the cells

Viability of cells was monitored with different concentrations of alginates after stimulation for 48 h, in an attempt to assess if there is any residual toxic effect of the compounds used. Every experiment was performed in double and representative results were reported in Fig. 1. Cell viability did not differ among negative control and cells stimulated with the lowest concentration of alginate. The sole exception was the highest concentration of alginate, which induced a significant reduction of cell viability.

Production of NO and TNF-α

In the first set of experiments we evaluated the effect of different concentration of alginates in the productions of NO and TNF-α in resident peritoneal monocytes, after different times. Every experiment was performed in double and representative results were reported in Figs. 2 and 3. Alginate was able to induce a dose-dependent production of NO after 24 h of stimulation. From our data, it seems that when cells were stimulated with 1.3 or 2.6 mg/mL, they produced similar level of NO (34.4 ± 16.3, and 31.2 ± 6.8 micromolar, respectively). implying that the reaction reached the plateau phase. The same experiment was performed after a 48 h period of stimulation showing that, independently from the stimulating dose (0.37, 0.65, 1.3, and 2.6 mg/mL), similar production of NO was achieved (52.8 ± 7, 58.8 ± 7, 54.4 ± 5.8, 51.2 ±7 .3 micromolar, respectively). Cells unstimulated produced a low amount of TNF-α (79 ± 8 pg/mL). Cells, stimulated with alginate for 48 h showed substantially similar pattern of TNF-α, irrespectively from the dose used (1146 ± 12, 865

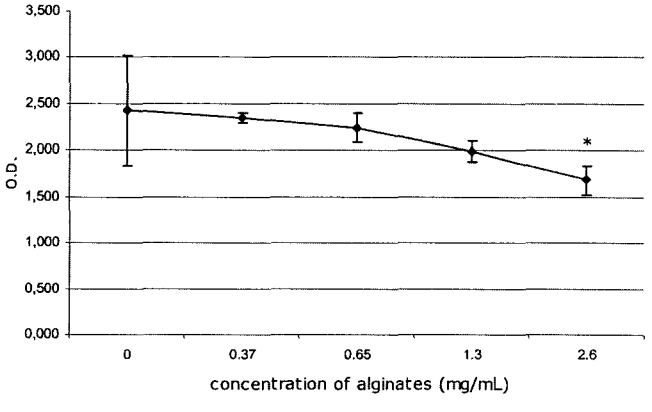

Fig. 1. Viability of peritoneal monocytes. Cells were stimulated with different concentration of alginates. After 48 h the viability of cells was measured through the formazan product. Data are the mean (SD) of three or four replicate cultures, and they refer to one of two performed experiments with comparable results. Values with asterisk are statistically different to values obtained from unstimulated cells (P≤0.05).

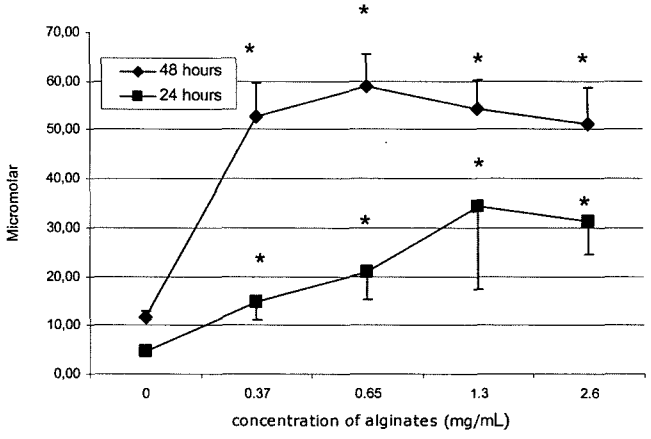

Fig. 2. Nitric oxide production. Peritoneal monocytes were stimulated with different concentration of alginates for 24 or 48 h. After incubation NO production was assessed by Griess reaction. Data are the mean (SD) of three or four replicate cultures, and they refer to one of two performed experiments with comparable results. Stimulated cells gave a significant increase of NO production irrespective to the concentration used (P≤0.05).

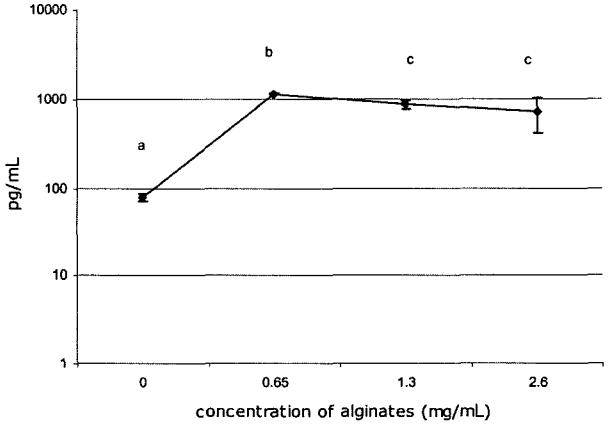

Fig. 3. TNF-α production. Peritoneal monocytes were stimulated with different concentration of alginates for 48 h. After incubation, supernates were harvested and TNF-α production was assessed by ELISA. Data are the mean (SD) of three or four replicate culture, and they refer to one of two performed experiments with comparable results. Values with different letters are statistically different (P≤0.05).

± 89, and 715 ± 297 pg/mL after stimulation with 0.65, 1.3, and 2.6 mg/mL, respectively, Fig. 3).

Flow cytometry

Peritoneal monocytes where stimulated with two different doses of alginate, choosing those which showed to be able to stimulate cells, without any toxic effects. Results are reported in Fig. 4. The percentages of CD80+ cells showed an increase associated to alginate stimulation, with little differences according to dose (48.6%, 52.5%, and 57.4% for cells unstimulated or stimulated with 0.65 and 1.3 mg/mL, respectively). The percentage of CD86+ cells showed a quite different pattern with a less clear difference between treated and untreated cells. In analogy with CD80+, also CD86 expression did not show any dose dependent effect, showing similar results obtained after stimulation with different concentration of alginates. The analysis of fluorescence intensity did not showed any difference between treated and untreated cells, irrespectively to the molecule.

Phagocytic efficacy

The results of phagocytosis are depicted in Fig. 5. When stimulated with 1.3 mg/mL of alginates, peritoneal monocytes showed an higher degree of phagocytosis (89.4 ± 1.1%) compared to cells stimulated with 0.65 mg/mL (86 ± 3%) or to unstimulated cells (86 ± 2.4%). Similar degree of intensity was however observed between stimulated and unstimulated peritoneal monocytes, showing that alginate induced a higher number of cells to phagocyte bacteria without increasing their phagocyting capability.

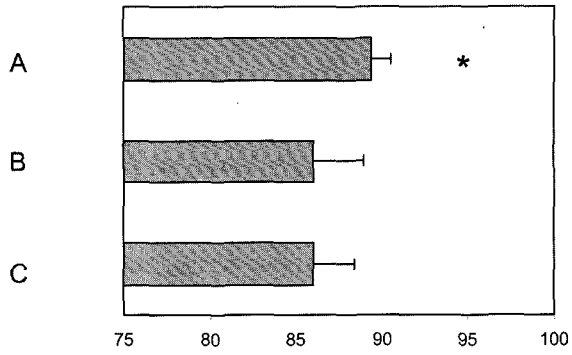

Fig. 5. Phagocytosis activity. Values refer to the percentage of phagocytosis in peritoneal monocytes, after stimulation with different concentration of alginates (A: 1.3 mg/mL, B: 0.65 mg/mL, C: unstimulated control cells). Data are the mean (SD) of three replicate cultures, and they refer to one of two performed experiments with comparable results. Values with asterisk are statistically different to values obtained from unstimulated control cells ($P \leq 0.05$).

DISCUSSION

We showed that alginate is able to increase proinflammatory functions of peritoneal monocytes. Monocytes play an important role in immune mechanisms, being particularly important as promoters of inflammation and as antigen presenting cells to T lymphocytes. Monocytes act in the immune response in a complex and multifaced way. Initially they are able to recognize foreign antigens and organisms, being able to phagocytise them. Successively, they are able to promote the inflammatory response through the secretion of pro-inflammatory cytokines. On

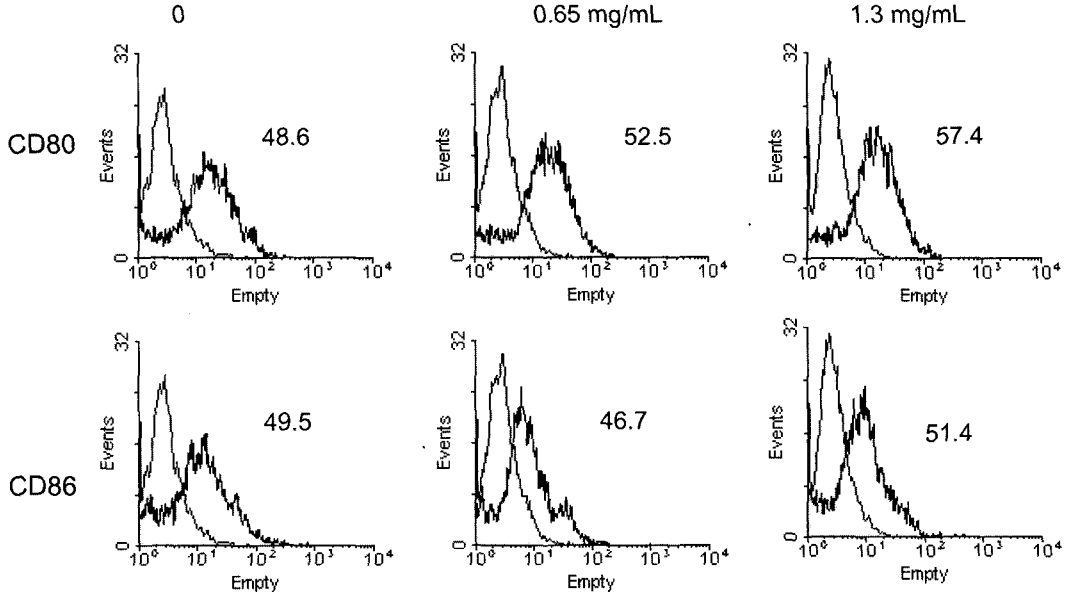

Fig. 4. Expression of CD80 and CD86 markers in peritoneal monocytes. Values refer to the percentage of cells positive for the different markers subtracted by the isotype controls (shadowed areas). Data are representative of two separate experiments.

the same time, they also interact with T lymphocytes as antigen presenting cells, regulated by co-stimulatory molecules. In this trial we focused on the effect of alginates on these crucial cells of immune system. In fact, the use of alginate as vehicle for antigens is well known but its direct effect is still not completely understood.

The use of alginates as adjuvants is being now proposed in order to substitute conventional adjuvants (i.e. mineral oils and Aluminium hydroxide), to guarantee the sustained release of the immunogens (Cho *et al.*, 1998), or to allow the oral way for vaccines administration (Bowerestock and Martin, 1999). It has been known that alginates are able to exert an immunostimulating activity in different experimental models. High mannuronic acid-containing alginate increased the number of peritoneal macrophages, phagocytosis, and production of nitric oxide (NO), hydroperoxide (H_2O_2), and TNF-α after *in vivo* exposure (Son *et al.*, 2001). In addition, alginates increased TNF-α, IL-1, and IL-6 production in *in vitro*-stimulated human monocytes (Otterlei *et al.*, 1991, 1993) in a pathway involving membrane CD14 and LPS binding protein (LBP, Espevik *et al.*, 1993) and soluble CD14 (Gullstein Jahr *et al.*, 1997). Here we demonstrated that alginates are effective also in *in vitro* stimulated mouse peritoneal monocytes, being able to induce the production of pro-inflammatory cytokines.

Antigen specific T cell immune response is primed by interaction between T cell receptor on T cells and antigens expressed on major histocompatibility complexes (MHC) II on antigen presenting cells. Additional signals, however, can modulate this interaction playing a costimulatory signal. Of those two receptors, namely CD80 and CD86, seem to play a crucial role in the development of T cell immune response (Sansom *et al.*, 2003). Initially they were believed to act similar effect, being able to bind to the same ligand on T cells. Successively, it has been found that CD80 and CD86 may differently play in T cell activation. In fact, treatment which downregulate CD86 functions results in a Th1-like response, suggesting that co-stimulation may play a crucial role in the differentiation of Th0 cells into Th1/Th2 response (Kuchroo *et al.*, 1995). We found that alginate was able to increase the percentage of CD80. Even if it is difficult to say if the increase of expression of costimulatory signals we observed can have any biological significance, it is intriguing because it reflects the ability of alginates to modulate those receptors that are directly involved in the antigen presenting activity of monocytes.

Due to their acidic polysaccharidic compositions, alginates mechanism of action could be similar to those of LPS complexes exposed on bacterial walls. The differences with respect to their *O*-acetylation and epimerization as well in ratio between mannurate and guluronate components of alginates are key determinants in conditioning structure-activity relationships (Gacesa, 1998). In conclusion, these data support and extend previously published data regarding to the effect of alginates on the functionality of immune cells and provide further information about their effect on the antigen presenting patterns. Such *in vitro* results confirm the practicability of this approach in boosting immuno-defences, with special emphasis to species and infections where the aspecific immunity could play a predominant role (i.e. fish or parasitic diseases) and highlight the need of a deeper comprehension of the complex and articulated effect of these molecules on the immune system.

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