Analysis of IL-2, IFN-γ and TNF-α production, α5β1 integrins and actin filaments distribution in peritoneal mouse macrophages treated with homeopathic medicament

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SUMMARY - The newer forms of Immune modulatory therapy are aimed at specific cells or cytokines that contribute to the immune response. These forms immunotherapy have been referred to as 'biological response modifiers'. Our lab was interested in investigating if a homeopathic medicament "Metodo Canova" (MC), sold in homeopathic drugstores, does enhance immunological system responses acting through macrophages pathway. Mice peritoneal macrophages were cultivated with or without homeopathic medicament for 24 h for α5, β1 and actin filaments distribution analyses through immunolabelling for confocal microscopy. To detect the IL-2, IFN-γ and TNF-α production these cells were cultivated for 48 h with or without medicament, followed by analyses of these cytokines in supernatant culture with ELISA kits. It was observed differences in morphology and molecular distribution (α5 and β1 integrins, actin filaments and Fc receptors) between the groups control and treated with MC. In control group macrophages had the morphology of resident cells and in MC treated group macrophages were more spread, had many cellular projections and a substantial increase in cytoplasmic volume. In addition, macrophages culture with two doses of MC showed that TNF-α production decreased when compared with control group.

INTRODUCTION

Specific immune system is induced or stimulated by exposure to foreign substances. Therapy directed at these immune responses can be either nonspecific or cell cytokine specific. The newer forms of immune modulatory therapy are aimed at specific cells or cytokines that contribute to the immune response. Therapy may also be directed to enhancing the individual's own immunity to favor a particular immunologic response. These forms of immunotherapy have been referred to as 'biological response modifiers' (Ballow and Nelson, 1997). Macrophages influence almost all aspects of immunologic and inflammatory responses and play an essential role in linking innate and acquired immune system (Unanue, 1997). A tissue macrophage lives for approximately 2-4 months.

During this time, some macrophages remain immobile, whereas others wander incessantly by amoeboid motion. In either case, the cell continually samples its surrounding environment by pinocytosis and through an extensive array of receptors on its surface. Whenever it encounters certain stimuli, the cell undergoes a process known as macrophage activation, in which it rapidly increases its metabolic race, motility, and phagocytic activity (Nathan *etal.,* 1998).

The functions of CD13/aminopeptidase N are not clear, although its abundance in the intestines and kidneys suggests its involvement in digestion of proteins for absorption. Because aminopeptidase N preferentially removes small neutral amino residues from N terminus of oligopeptides, it has been suggested that it has a physiologic role, other than in the nutritional protein degradation process, which is to inactivate certain biologically active peptides (Kenny *et al.*, 1989). In mouse, Chen *etal.* (1996) observed that among hematopoietic lineage cells, expression of CD 13 is restricted to mast cells and some macrophages. These cells are deeply involved in inflammation and allergic responses through their release of histamine and other bioactive peptides. The results obtained strongly indicate that mouse pi 61 is CD13/aminopep tidase N. In addition, Kinzer *et al.* (1995) described the derivation of a mAb (K-l) specific for a 161-kD membrane protein (pl61) expressed on mouse mast cells and on some macrophages but not on other hematopoitic cells.

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The rapid increase in the number of cytokines and growth factors that have been shown to modify macrophages functions has widely demonstrated the complexity of macrophage activation; for instance, many of the cytokines involved are also produced by macrophages and are likely to have autocrine effects (Erwig, 1998). The proinflammatory cytokines including some interleukins and TNF-α play an important role in the pathophysiology of a number of inflammatory diseases. Several of the interleukins can be used as adjunct therapy in patients undergoing cancer treatment or as bone marrow recovery-enhancing reagents following chemotherapy and transplantation. Clinical studies have concentrated on IL-2, a potent immunoregulatory cytokine. The interferons — IFN-α, IFN-β, and IFN-γ — comprise a unique category of biologically active proteins that have antiviral activity (Ballow and Nelson, 1997). In addition, interferons play an important role in resistance to tumors, the control of cell growth and differentiation, the expression of cell surface molecules, and the modulation of immune responses (Balkwill, 1989). IFN-γ has been shown to play a key role in host defense by exerting antiproliferative, immunoregulatory, and proinflammatory activities (Farrar and Schreiber, 1993).

The plasma membrane receptors control macrophage activities such as growth, differentiation, activation, migration and secretion. The principal receptors binding the extracellular matrix compounds are integrins that communicate with cytoskeleton molecules. The integrins are family of transmembrane glycoproteins that are expressed by the cell as $\alpha\beta$ heterodimers (Hynes, 1992). A common β-subunit can associate with several unique α subunits to form functionally distinct receptors. Integrin-ligand interactions are accompanied by activation and clustering of integrins on the cell surface and transduction of this activation into intracellular signal transduction pathways that mediate a variety of intracellular events (Burridge *et al.,* 1992; Dash *et al.,* 1995; Kohn *et al.,* 1995). Members of the (β1 subfamily of integrins mediate the attachment of monocytes and macrophages to cell matrix proteins such as fibronectin, collagen, and laminin. Such interactions are likely to have considerable importance during inflammatory response, when monocytes are recruited to, and retained in, extravascular sites (Kohn and Klingemann, 1991). Fibronectin besides collagen, is a major component of the extracellular matrix. It plays a major role in cell adhesion and spreading, cytoskeletal organization, cell differentiation, tissue remodelling, and morphogenesis in early vertebrate development (Grimell, 1978; Darribcre *et al.,* 1992). This glycoprotein also plays a crucial role in wound repair, stimulating the adhesion, proliferation and motility of cells migrating into the wound (Clark *et al,* 1982).

Many cell types are able to bind circulating antibodies or antigen-antibody complexes using surface Fc receptors. Fcγ receptor (FcγR) is an integral membrane protein that specifically binds to the Fc portion of IgG proteins at the surface of various cells including macrophages, monocytes, neutrophils, natural killer (NK) cells, and B and T cells (Suzuki, 1991).

Today, in Brazil and Argentina, at homeopathic drugstores, a product indicated to patients with depressed immunologic system is sold. This product, named Metodo Canova (MC), contains as fundamental substances: *Aconitum, Bryoma, Thuya, Lachesis* and *Arsenicum album* (information obtained from the homeopathic manipulator). Our lab was interested in investigating if this product does enhance immunological system responses acting through macrophages pathway.

MATERIALS AND METHODS

Macrophage culture onditions

Resident macrophages were collected from peritoneal cavities of normal Swiss mice from the Animal Facilities at Federal University of Paraná. Immediately after killing the animals with ether, their peritoneal cavities were washed with PBS, pH 7.4 and the cells were plated on plastic flasks or on glass coverslips. After incubation for 15 min at 37 °C, the non-adherent cells were removed and DMEM medium with 10% fetal calf serum (PCS) and 2.2 g/l glutamine was added. After incubation at 37 °C, 5% CO_2 for 24 h or 48 h, with or without 10% MC, macrophage cultures were rinsed with PBS and used for the experiments. *In vivo* experiment: the treated group animals were inoculated subcutaneously during seven days with 7 pl/g MC. Control group animals were inoculated with sterile PBS, pH 7.2. After treatment macrophages were collected, cultivated for 24 h and processed for optical microscopy (repeated five rimes). *In vitro* experiment: the macrophages were cultivated in 3 different conditions: *a)* in presence of the MC or PBS, pH 7.2, solution containing 0.01% ethanol; *b)* incubated with different concentrations of Canova 200: 2, 5, 10 or 20% for 24 h; *c)* control groups were cultivated without medicament (repeated five times).

To be counted, the cells were divided into two groups: activated and resident macrophages. To determine activated macrophages, morphological characteristics were considered: big nucleus, more euchromatin (bright) than heterochromatin (dark), spread, many microvilli or projections and vesicular cytoplasm. A hundred macrophages were counted in each slide.

Light microscopy

Macrophages were fixed for 5 min in Bouin's fixative, stained with Giemsa, dehydrated in acetone, and slides were mounted with Entelan and observed in Olympus BHS microscope.

Scanning electron microscopy

The macrophages were plated on coverslips, incubated with 10% Canova for 21 h in vitro, fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, dehydrated in ethanol, critical point dried in CO_2, coated with a thin layer of gold and observed in scanning electron microscope Philips XL30.

Confocal microscopy immunofluorescence

Primary antibodies - MoAb purified rat anti-mouse CD49e (Integrin a5 chain), MoAb purified rat anti-mouse CD29 (Integrin b1 chain), MoAb purified rat anti-mouse CD16/CD32 (Fc-gIII/II Receptor), MoAbs nuorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD 13, rabbit anti-mouse anti-Fn polyclonal antibody, IgG anti-mouse FcR, Phalloidin-Texas Red.

Secondary antibodies - Fluorescein (FITC)-conjugated affinipure F(ab')2 Fragment donkey anti-rat IgG (H + L), fluorescein (FITC)-conjugated affinipure F(ab')2 Fragment donkey anti-rabbit IgG (H + L), fluorescein (FITC)-conjugated affinipure F(ab')2 Fragment donkey anti-mouse IgG (H + L). After 24 h of culture macrophages treated with 10% MC for 21 h and control group were processed for different protocols:

a) for CD13 immunostaining macrophages first were fixed in 2% paraformaldehyde/methanol for 5 min at -20 °C, and incubated with 1 mg/ml CD16/CD32 (FcgIII/II Receptor) to block Fc receptors, and the FITC-conjugated anti-CD13 (15 mg/ml); b) to show Fc receptors at macrophage surface, the cells were incubated in 5 mg/ml mouse IgG diluted in culture medium for 1 h at 4°C and then fixed in 2% paraformaldehyde/PBS, pH 7.2, for 30 min, to be revealed the cells were incubated with secondary antibody FITC-conjugated F(ab')2 anti-IgG (25 mg/ml); c) to detect a5 and b1 integrin and fibronectin at the cell surface, the cells were fixed for 30 min in 2% paraformaldehyde/PBS, pH 7.2, rinsed in 0.1 M glycin/PRS and in 1% BSA/PBS. Then macrophages were incubated with primary antibodies: anti-a5 (5 mg/ml), anti-b1 (25 mg/ml), anti-Fn (1:20) in 1% BSA/PBS for 1 h, after that incubated with respective secondary antibodies (25 mg/ml) for 30 min in the dark; d) fibronectin receptor a5b1 integrin function was detected by incubating macrophages with fibronectin (5 mg/ml) in culture medium for 1 h at 4 °C, fixing in 2% paraformaldehyde/PBS, pH 7.2, for 30 min followed by incubation with anti-Fn (1:20) in 1% BSA/PBS for 1 h. Incubation with the secondary antibody, FITC-conjugated F(ab')2 anti-IgG (25 mg/ml) in PBS, pH 7.2, for 30 min;
e) to label actin filaments, Phalloidin-Texas Red (25 mg/ml) was used for 30 min after fixation and permeabilization with 0.01% Triton X-100/PBS, pH 7.2, for 5 mm; f) for a5 and actin filaments double labelling, the cells were fixed for 30 min in 2% paraformaldehyde/PBS, pH 7.2, rinsed in 0.1 M glycin/PBS and in 1% BSA/PBS. Then macrophages were incubated with primary antibody anti-a5 (5 mg/ml), in 1% BSA/PBS for 1 h, after that incubated with the respective secondary antibodies (25 mg/ml) for 30 min in the dark. For actin filaments labelling, the cells were rinsed in PBS, pH 7.2, permeabilized with 0.01% Triton X-100/PBS, pH 7.2, for 5 min and incubated with Phalloidin-Texas Red (25 mg/ml) for 30 min; g) negative controls were incubated without primary antibody. The slides were mounted with Fluormount and observed in a Leika confocal microscope.

ELISA - Enzyme-linked immunosorbent assay

Mouse IFN-g, TNF-a and IL-2 immunoassay (Quantikine ™Murine Kit for research - R&D Systems). This assay aimed at quantitative determination of mouse interleukin 2, Tumoral Necrosis Factor-a or Interferon-g concentrations in cell culture supernatant, employing quantitative sandwich enzyme immunoassay technique. For ELISA macrophages were cultivated a) for 48 h with addition of one dose (100 ml/ml) of MC after 3 h in culture and b} with two doses: first 100 ml/ml after 3 h and 50 ml/ml after 24 h in culture. After 48 h in culture, 15 ml of supernatant is concentrated at 0.5 ml with 10 kD centrifugal concentrator filter (Macrosep™ Pall Filtron) in a fixed-angle centrifuge at 5,000 rpm for 45 min, and processed in conformity with kit protocols. The optical density is measured at 450 nm. The samples values are read off the standard curve. The samples values were read in an ELISA Reader Behring EL-311 (Marburg, Germany).

RESULTS

In vivo experiment

Mice macrophages treated with MC show 86% of macrophage activation and 15% control group (Fig. 1). Macrophages were considered activated when some morphological characteristics were present: big nucleus, more euchromatin than heterochromatin, spreading, vesicular and clear cytoplasm with many microvilli or projections (Fig. *2a* control group and Fig. 2*b* treated group).
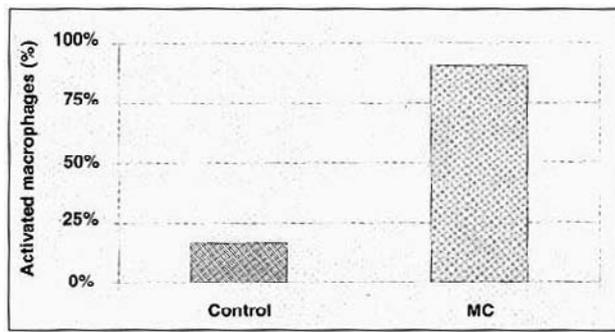
FIGURE 1 *In vivo* experiment. Difference in percentage of activated macrophages between treated (86% activated macrophages) and control group (15% activated macrophages) is shown.
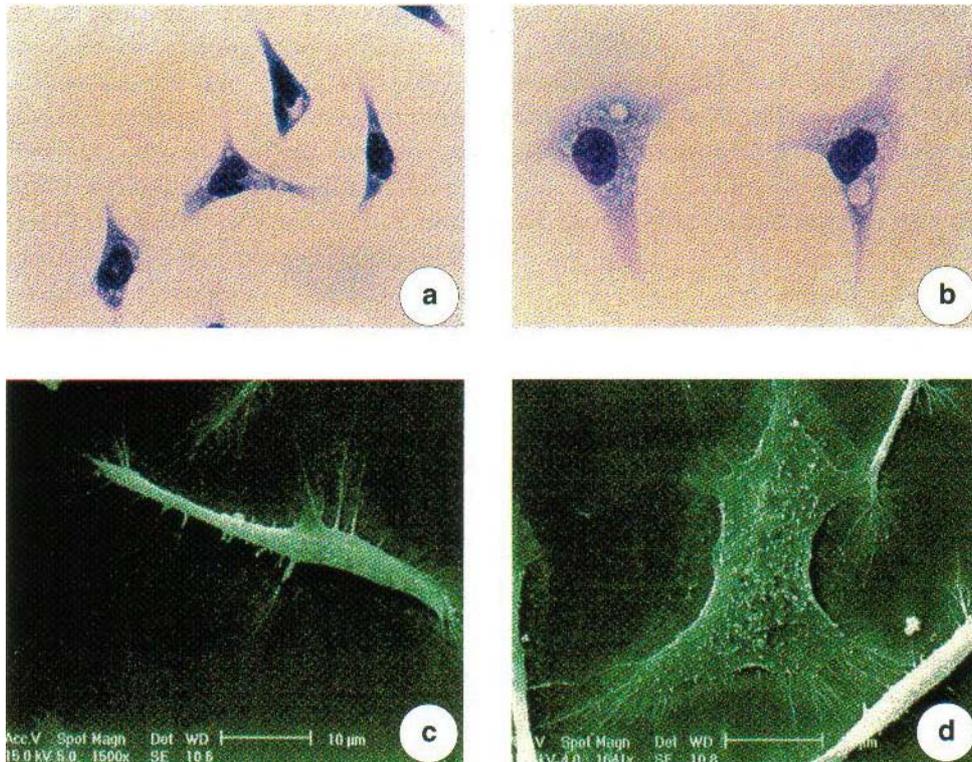

FIGURE 2*a-d* Microphotographs and scanning electron micrographs of macrophages. *(a,b)* Microphotographs of control and treated macrophages respectively, at optical optical microscopy. *(c,d)* Scanning electron micrographs of control and treated groups. In control one can see little and condensed nucleus, few cytoplasmic projections and in treated group one can observe morphological characteristics of activated cells: spread, big nucleus, more euchromatin, vesicular and clear cytoplasm and many projections. X 1,000.

In vitro experiment

The number of activated macrophages increased proportionally with MC concentration. With concentrations of 1, 2, 5, 10 and 20% the percentages of activated cells were 23, 55, 55, 72 and 81% respectively. Control group showed activation of 33% (Fig. 3). Ethanol group showed 34% of activated macrophages, that were cultured with 10% of the ethanol 0.01% solution (data not shown).
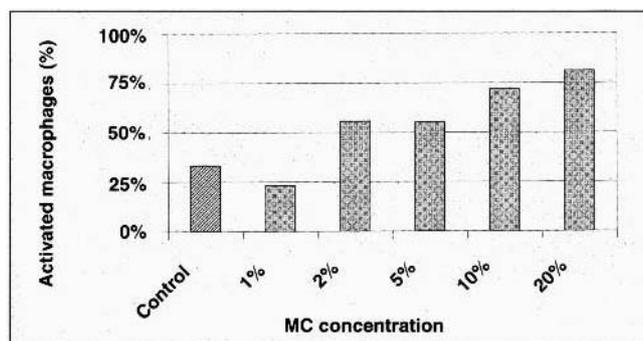

FIGURE 3 *In vitro* experiment to detect difference in percentage of activated macrophages cultivated with different MC concentrations. It shows different quantity of activated macrophages cultivated with 1, 2, 5, 10 and 20% of MC with respectively 23, 55, 55, 72 and 81% of activated macrophages.

Scanning electron microscopy

Observations of macrophages after adherence to glass surface and treatment with Metodo Canova in culture showed that the major cells were activated, spread, with many filiform projections resembling short microvilli while resident macrophages cultivated without medicament did not show these morphological characteristics (Fig. *2c,d*).

Confocal microscopy immunofluorescence

Images of confocal microscopy immunofluorescence can be observed in Figs. *4a-h* to *6a-i*. Differences in morphology and molecular distribution were observed between the groups: control, ethanol treated and MC treated. In control group macrophages had the morphology of resident cells, with few cellular projections and not well spread. In ethanol group macrophages had an intermediary morphology, most macrophages were resident and some had the same mor phology as the activated cells. In MC treated group macrophages were more spread, had many cellular projections and a substantial increase in cytoplasmic volume.
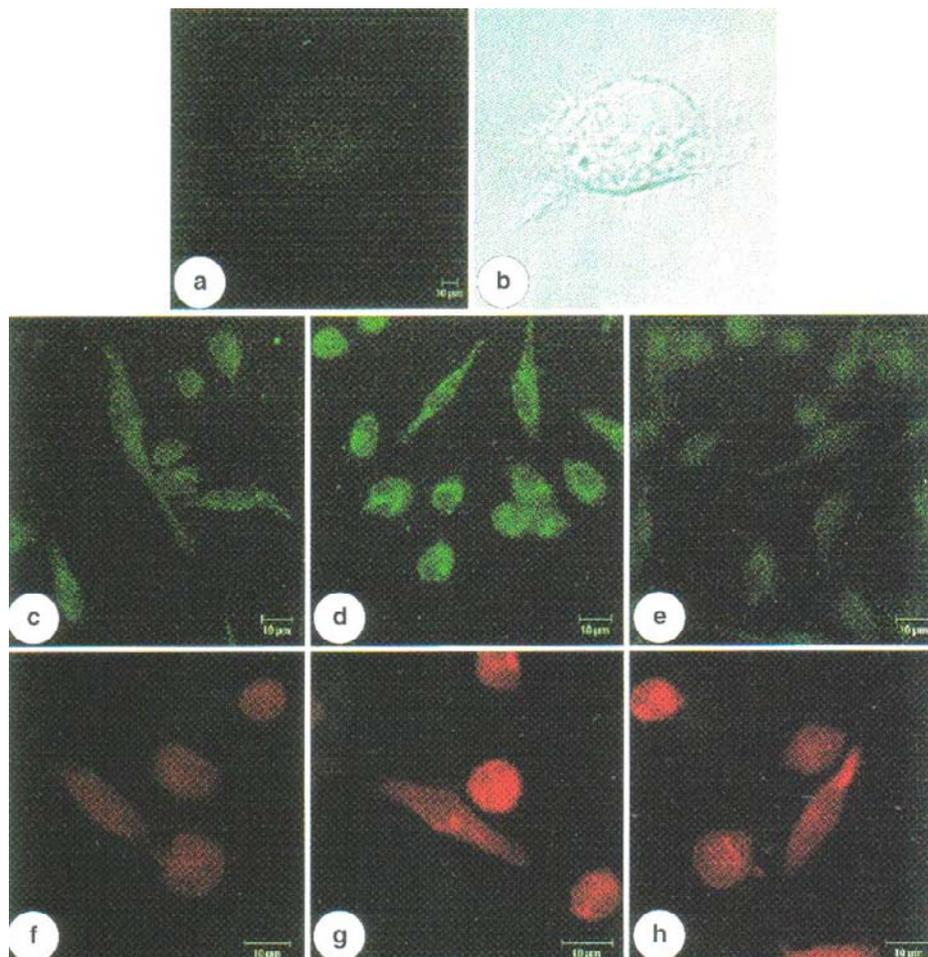

FIGURE 4*a-h* Negative control *(a)* confocal microscopy and *(b)* bright field microscopy. Immunostaining to CD13 and Fc receptors. The positive result to CD13 demonstrated there were only peritoneal macrophages, *(c)* control, *(d)* ethanol and *(e)* treated group. All groups were positively labeled for Fc receptor. In *(f)* one can see control group with uniform distribution of receptors and in ethanol *(g)* and treated *(h)* groups the receptors were clustered.

All control groups, incubated without primary antibody, showed a negative immunostaining (Fig. *4a,b*). The positive results for CD13 label demonstrated there was only peritoneal macrophages in culture (Fig. *4c-e*). All groups were positively labeled for Fc receptors, but in ethanol and MC groups the receptors were clustered (Fig. *4f-h*).

No differences were observed on label of α_5 integrin with con-focal microscopy (Fig. *5a-c*). β_1 integrin labeling showed a clustered distribution in ethanol and MC groups (Fig. *5d-f*). Fibronectin receptor $\alpha_5\beta_1$ integrin was functional and positively labeled in all groups (Fig. *5g-i*). Actin filaments were positively labeled at confocal microscopy in all groups, showing a peripheric distribution and a substantial increase in number of actin rich microspikes and protrusions in MC group (Fig. *5j-l*).

Double labeling of α_5 (green) and actin filaments (red) showed a coincident label in cellular periphery in all groups. In treated group (Fig. *6g-i*) it can be observed an evident intracellular ring of actin filaments, many microspikes and several protrusions. Fig. *6a-c* shows images of control group; Fig. *6d-f* shows ethanol group.
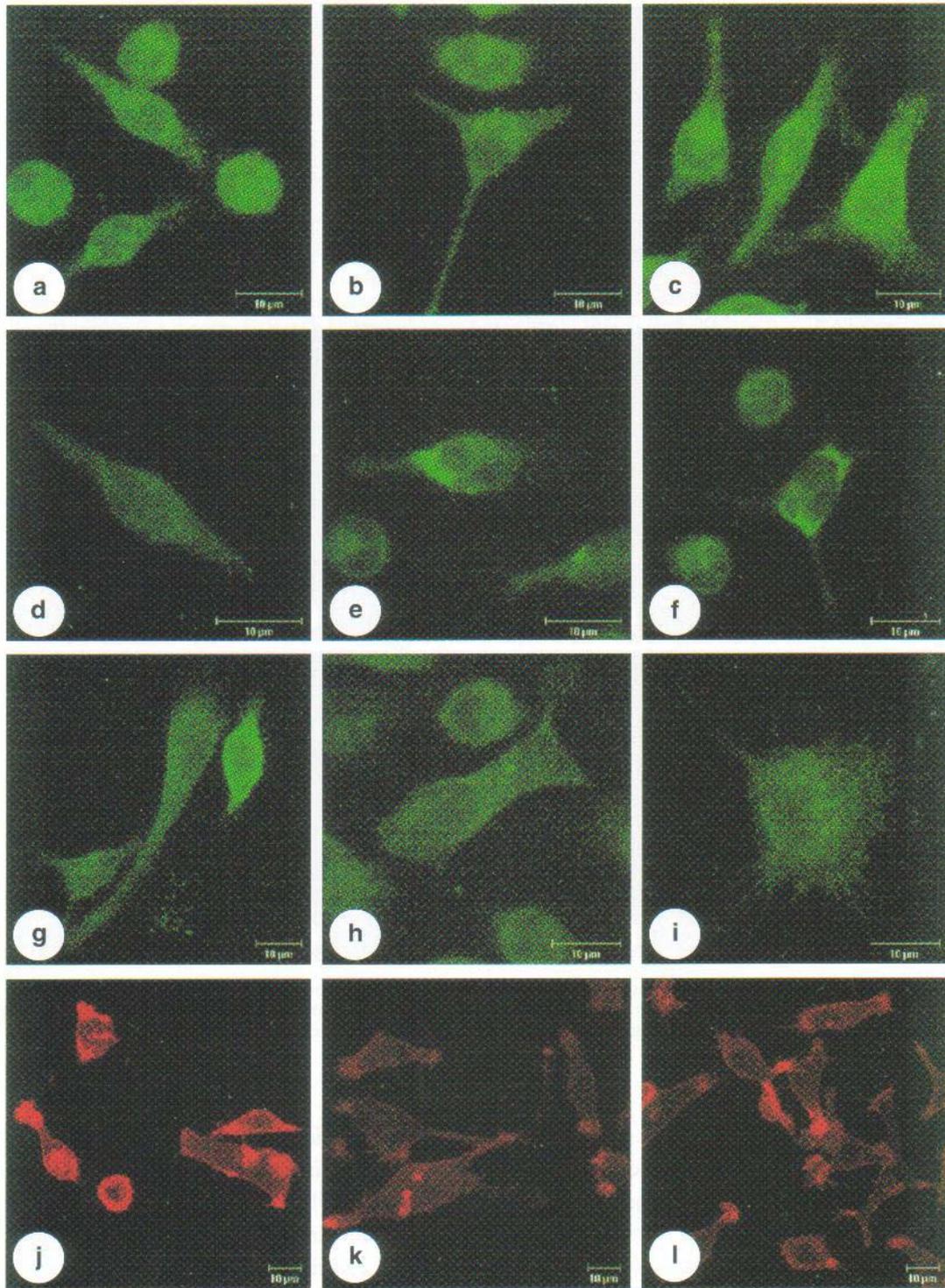
FIGURE *5a-l* Immunolabeling to α_5, β_1 integrin, fibronectin receptor α_5 β_1 integrin and actin filaments. No differences were observed in label of α_5 integrin, (*a*) control, *(b)* ethanol and *(c)* treated. β_1 integrin label showed a clustered distribution in ethanol *(e)* and treated (*f*) group, *(d)* control. Fibronectin receptor α_5, β_1 integrins were functional and positively labeled in all groups, *(g)* control, *(h)* ethanol and (*i*) treated. Actin filaments were also positively labeled in all groups. In *(k)* ethanol and in (*l*) treated groups one can see a peripheric distribution and a substantial increase in number of actin filaments, many microspikes and protrusions. Control group (*j*).
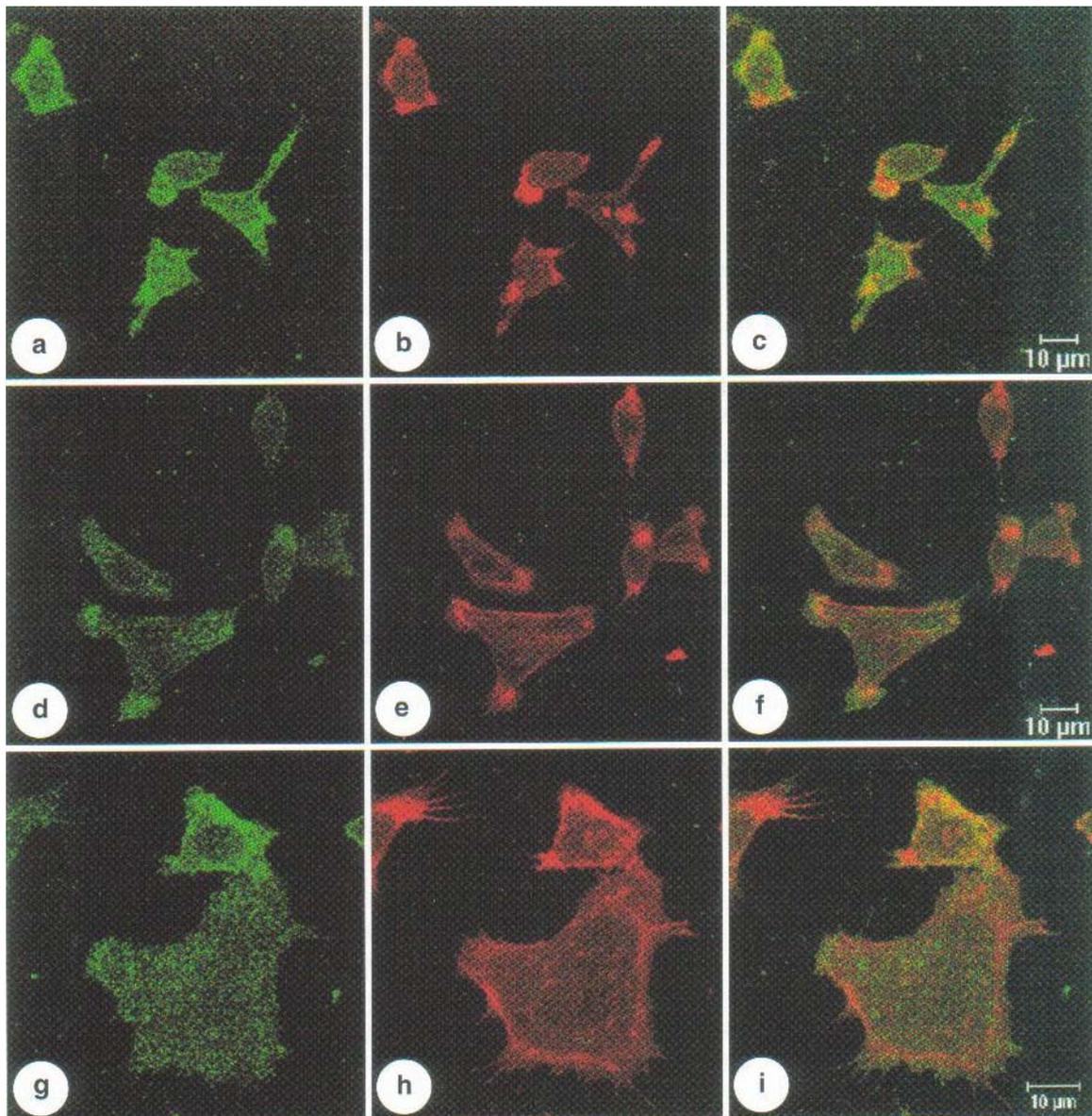
FIGURE 6a-i Double immunolabeling to α_5 integrin and actin filaments. Conrrol group: *(a)* α_5 integrin, *(h)* actin filaments and *(c)* the two labels superposed. Ethanol group: *(d)* α_5 integrin, *(e)* actin filaments and *(f)* the two labels superposed; both, control and ethanol groups, show a coincident label in cellular periphery. Treated group: *(g)* α_5 integrin, *(h)* actin filaments and *(i)* the two labels superposed; one can observe a coincident label in cellular periphery and an evident intracellular ring of actin filaments, many microspikes and several protrusions.

ELISA

IL-2 and IFN-γ showed no difference between treated and control group. TNF-α production also showed no difference when only one dose was added. Macrophages cultured with two doses of MC showed that TNF-α production decreased when compared with control group. It was detected 1,350.2 pg/ml at the control group while at the treated group the TNF-α concentration was 290 pg/ml (Fig. 7).

DISCUSSION

Positive immunolabeling for CD13 molecules showed that in cultures with 15 min of adherence only macrophages were present. Various cytokines are known to affect the level of FcγR expression. Less is known about the effector functions of individual FcγR classes following specific homeopathic treatment. Positive or negative effects of a cytokine on a single cell type may be related to differences in state of maturation, differentiation, or activation (Van Hal *et al.,* 1992). Transmembrane signalling by FcγRs should lead to a substantial modulation of macrophage functions. In addition to a role in cell attachment and adhesion, binding of fibronectin to its receptor(s) on macrophages may have functional consequences, and fibronectin has been found to increase the phagocytic activity of macrophages, presumably as a result of an enhancement of complement (C3) receptors (Pommier *et a/.,* 1983), and to active receptors for Fc portion of IgG (Wright and Meyer, 1985). Monocytes and macrophages attachment to fibronectin present in the subendothelium or deposited at sites

of inflammation is also mediated, at least in part, by $\alpha_5\beta_1$ expressed on the surface of these cells. If this is the case, $\alpha_5\beta_1$ would play an important role in the retention of monocytes/macrophages at these sites (Kohn and Klingemann, 1991). Wojciak-Stothard *et at* (1997) showed that both fibronectin and fibronectin fibers stimulated the polymerization of F-actin content in macrophages and that the F-actin was concentrated at the leading edge of the cell in areas of cell attachment to the substrate and in numerous microvilli. Our results related with fibronectin, $\alpha_5\beta_1$ integrins and actin filaments suggest that this medicament induces mouse peritoneal macrophages to adapt an activated aspect as well as morphologic and molecular redistribution of fibronectin in surface, α_5 integrin, β_1 integrin and actin filaments.
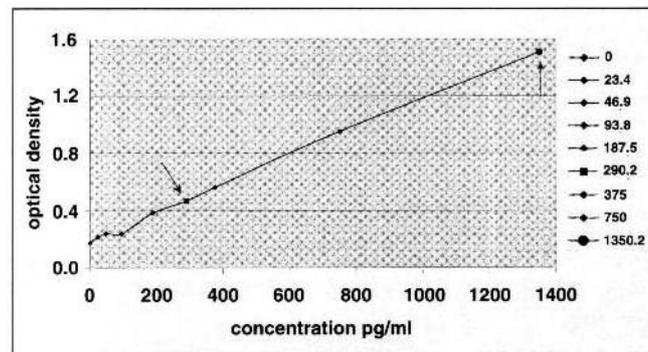

FIGURE 7 ELISA to TNF-α. TNF-α standard curve showing decreased production of TNF-α in macrophages treated with MC ($\rightarrow\square$). Treated group 290 pg/ml and control group ($\rightarrow\bullet$) 1.350.2 pg/ml.

Recently, considerable interest has focused on a family of cytokines, polypeptides produced by lymphocytes or macrophages as part of the inflammatory reaction to stress, infection or malignancy. These cytokines appear to have metabolic effects related to tissue wasting and therefore may play a role as mediators of the overall response. Until recently, IL-2, IFN-γ and TNF-α were measured by bioassays, which are susceptible to interference from other constituents of biological fluids. The development of enzyme-linked immunosorbcnt assay (ELISA), used in this work, or radioimmunoassays for the determination of the cytokines should give more information on their concentrations in body fluids or production by isolated cells or tissues (Evans *et al.,* 1989).

Erwig *et al.* (1998) showed that the functional activation of uncommitted bone marrow-derived macrophages is determined by the first cytokine to which the cells are exposed; once the activation process has started, macrophages become temporarily unresponsive to alternative stimuli. The initial studies reported by Rosenberg *et al.* (1985), from the National Institute of Health used IL-2 for the treatment of cancers that were resistant to chemotherapy. IL-2 enhances the cytotoxic function of lymphocytes, lymphokine-activaced killer (LAK) cells to a broad range of tumor cells. Unfortunately, IL-2 has a significant toxic effect, involving every organ system, particularly when used at higher doses. IFN-γ has been approved for the treatment of cell leukemia, condyloma acuminata, acquired immunodeficiency syndrome (AIDS)-related Kaposi sarcoma, hepatitis B, and hepatitis C (Dorr, 1993). Many investigations have confirmed that IFN-γ is a potent activator of monocytes/macrophages and can enhance tumoricidal activity of mononuclear phagocytes, particularly in models which examine ADCC (antibody-dependent cellular cytotoxicity). The augmented ADCC after IFN-γ priming of macrophages correlates with the up-regulated FcR expression (Kurzrock *et at,* 1991). The adverse effects of interferon include flulike symptoms of fever, chills, headache, myalgia, arthralgia, fatigue, and anorexia, usually seen in the initial phases of treatment. High-dose therapy with interferon may lead to hematologic toxic effects including anemia, leukopenia, and thrombocytopenia, as well as gastrointestinal toxic effects. Abnormal liver functions test results can also be seen as an adverse effect of interferon therapy (Ballow and Nelson, 1997). Our results related with IL-2 and IFN-γ suggest that this medicament did not interfere with release of these cytokines.

Macrophages vary in respect to priming for TNF release and heterogeneity should be related to a particular triggering stimulus, and the capacity of some macrophages populations to release unusually high levels of TNF depends on immune or nonspecific stimuli subsequent to the process of inflammatory recruitment (Stein and Gordon, 1991). Phagocytic plasma membrane receptors utilize different pathways to trigger TNF release, which depends on selective ligation of specific receptors, such as FcR. Release of active TNF is therefore heterogeneous, although closely regulated in different macrophages, by a range of distinct inflammatory, immune and endocytic signals (Stein and Gordon, 1991). TNF-α is an important mediator in the pathogenesis of sepsis syndrome and septic shock. Animals' studies have shown that infusions of neutralizing anti-TNF antibodies prevent shock and death due to bacteremia or endotoxemia (Dinarello *et al.,* 1993). This experiment showed that excessive quantity can be prejudicial, leads to the appearance of many diseases, often malignancies and eventually to death. TNF-α and β play a critical role in normal host resistance to infection and to growth of malignant tumors, serving as immunostimulants and as mediators of the inflammatory response. Over-production of TNFs, however, has been implicated as playing role in a number of pathological conditions, including cachexia, septic shock, and autoimmune disorders. TNF-α induced a decrease in polymerized actin and an increase in micropinocytosis and inhibited Cdc42 - (induces the formation of filopodia in Swiss 3T3 cells) mediated filopodium extension (Peppelenbosch *et al.,* 1999). Our results showed that TNF-α release decreased after repeated doses of MC, suggesting a possible effect of this homeopatic

medicament. Resident peritoneal macrophage activation by Metodo Canova (MC) is strongly influenced by stimulation conditions such as MC concentration.

Our results point to the need for careful examination of the interplay between homeopathic medicaments and macrophages in the treatment of malignancy. As this knowledge is extended, the ability to selectively influence the activation state of the macrophage, hopefully will allow us to manipulate these treatments in the malignant disease. This will be an important area for future research, in particular the functional relationship between reticulo-endothelial cell production of cytokines and the response to homeopathic treatment.

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