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Sodium caseinate induces secretion of macrophage colony-stimulating factor from neutrophils

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Abstract

In this work we provide evidence that granulocytes produce macrophage colony-stimulating factor (M-CSF) in the band cell stage and secrete it upon sodium caseinate-mediated differentiation to polymorphonuclear cells. We identified M-CSF in an enriched population of myeloid band cells from murine bone marrow using a chromophore-labeled monoclonal anti-M-CSF antibody. An ELISA assay was then used to detect secreted M-CSF in culture supernatants of enriched band cells differentiated to mature neutrophils using sodium caseinate. Colony formation *in vitro* by the supernatants from differentiating band cells was blocked by anti-M-CSF, thus suggesting that this factor is the only one responsible for this activity. Our data imply that casein can modulate hematopoiesis possibly via M-CSF production. Finally we discuss the possibility whether this M-CSF in concert with G-CSF could establish a cellular communication network between macrophages and granulocytes allowing them to simultaneously arrive at the inflammatory site.

Keywords: Band cells; M-CSF production; Neutrophils; Sodium caseinate

Introduction

Sodium caseinate (CasNa), a salt of casein, the main protein of milk, was used more than 20 years ago to produce an inflammatory response on the peritoneal cavity of mice (Lotem and Sachs 1983). CasNa inoculation was performed intraperitoneally and cells were harvested at different times. Granulocytes appeared in great numbers at only 16 h after inoculation, followed several days later by monocyte and lymphocyte migration (Lotem and Sachs 1983, 1985).

Soon afterwards, several growth factors including macrophage-colony-stimulating factor (M-CSF), granulocyte-CSF (G-CSF) and granulocyte-macrophage-CSF (GM-CSF) were detected in high concentrations in the sera of mice treated with sodium caseinate or casein (Lotem and Sachs 1985; Metcalf et al. 1996); however, the cells producing these factors were not identified. It was from these sera that the first

Abbreviations: CasNa, sodium caseinate; M-CSF, macrophage-colony-stimulating factor; G-CSF, granulocyte-colony-stimulating factor; GM-CSF, granulocyte-macrophage-colony-stimulating factor; FBS, fetal bovine serum; PBS, phosphate-buffered saline; BMC, bone marrow cells.

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hematopoietic growth factors were discovered, purified and studied (Sachs 1990). Although recombinant growth factors are now available (Kaushansky 2006), inoculation with CasNa or casein has remained a method for inducing inflammation in the peritoneal cavity of mice (Metcalf et al. 1996).

Our laboratory has shown that CasNa inhibits the proliferation of myeloid cells and induces their differentiation along the monocyte-macrophage lineage (Ramos et al. 2000, 2004). Recently, we showed that alpha-, beta- and kappa-casein, as well as CasNa all inhibited 32D cl3 and WEHI-3 cell proliferation; however, only CasNa strongly induced secretion of M-CSF in 32D cl3 cells (Ramos-Mandujano et al. 2008). It was thus interesting to determine whether CasNa could induce M-CSF production in normal myeloid bone marrow cells.

Given that granulocyte-band neutrophils can secrete M-CSF (Santiago et al. 2001) and that intraperitoneal CasNa inoculation induces strong granulocyte accumulation (Lotem and Sachs 1983, 1985), in this work we evaluated whether M-CSF was secreted from granulocytes treated with CasNa.

Materials and methods

Mice

Bone marrow cells (BMCs) were obtained from strain CD-1 mice of either sex at 6–8 weeks of age.

Cytokines and antibodies

Recombinant human G-CSF (rhG-CSF), recombinant murine M-CSF (rmM-CSF), and goat IgG anti-murine M-CSF were purchased from R&D Systems (R&D Systems Inc, MN, USA). Peroxidase-labeled rabbit anti-goat IgG was purchased from DAKO (DAKO, MA, USA). Sodium caseinate was purchased from DIFCO Laboratories, Michigan, USA.

Cell culture

All BMC cultures were maintained under 5% CO_2 atmosphere at 37 °C and 95% relative humidity. The cells were cultured in RPMI-1640 medium (Gibco BRL, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco BRL, USA) previously inactivated at 56 °C for 30 min. Streptomycin (100 mg/mL), penicillin G (100 U/mL), and sodium bicarbonate (3.7 g/L) were added to the RPMI 1640 medium before culture. The cultures were prepared using either 6×10^6 cells in 10×30 mm dishes or 10^5 cells in 24-well tissue culture plates (Costar, Cambridge, MA, USA).

Cell lines

The CaLo cell line was derived from a stage IIB human cervical carcinoma; these are adherent cells with a high proliferation rate. The cells were grown in RPMI 1640 supplemented with 10% (v/v) FBS. L-929 cells are fibroblasts of murine origin and produce several cytokines including M-CSF. L-929 cells were cultured in IMDM medium (Gibco BRL, USA) supplemented with 10% (v/v) FBS.

Bone marrow cells (BMCs)

To prepare BMCs, RPMI-1640 medium was flushed through individual mouse femoral shafts using a syringe until bone marrow tissue was obtained. Cells were pooled and washed three times by centrifugation in RPMI-1640 medium, and then resuspended in 10 mL RPMI-1640 containing 10% (v/v) FBS.

Harvest of band cells

Collected BMCs were centrifuged in a density cushion of ficoll (1.077 g/mL) for 30 min at 500*g*. The cell pellet was washed twice by centrifugation in phosphate-buffered saline (PBS), and then centrifuged over a ficoll cushion (1.083 g/mL) for 20 min at 500*g*. The cells collected at the interface, consisting mostly of band cells, were harvested and washed 3 times in PBS for subsequent identification using an optical microscope, and cultured as indicated above. Under our experimental conditions, we obtained 85% band cells, 10% mature polymorphonuclear cells, 3% macrophages, and 2% lymphocytes.

Colony-forming assay

For the colony-forming assay, the double agar layer technique was employed to culture bone marrow cells (Pluznick and Sachs 1966). Briefly, a first layer with 0.6% (w/v) agar was added with RPMI 1640 medium, 20% (v/v) horse serum (HS) and the colony-stimulating factor (CSF) or inducer to be tested: these included 20 ng/mL rhG-CSF or 20 ng/mL rmM-CSF or 20% (v/v) supernatants and lysates from cultured band cells treated with or without 20 μL/mL sodium caseinate (10% in PBS). A second layer with 0.3% (w/v) agar, RPMI-1640 medium and 10% HS (v/v) was then overlaid with 10^5 BMCs per well. After 7 days of incubation, all colonies with more than 20 cells were counted using an inverted light microscope.

Production of supernatants and cell lysate

Band cells stimulated with 20 μL/mL of sodium caseinate (10% in PBS) and 20 ng/mL rhG-CSF for 24 and 48 h were collected; supernatants were collected as well. Cells were washed twice by centrifugation at 500*g* with RPMI-1640 medium and lysed by a double cycle of freeze–thaw using liquid nitrogen. RPMI-1640 was added to the lysate to generate a final concentration of 10^6 lysed cells/mL. Supernatants and cell lysates were stored at −20 °C until used.

Colony and cell morphology

Morphology of colonies in the double-layer agar was evaluated using a modification of a coverslip transfer technique (Moezzi et al. 1986). Briefly, the upper agar layer was cut into pieces of approximately 2.5 × 1 cm, and a sheet of dry Whatman No. 2 filter paper (Whatman Incorporated, Clifton, New Jersey, USA) of the same size was placed over the agar. As soon as the filter paper became moist, it was carefully removed. The attached colonies were transferred by placing the filter paper onto a coverslip for 45 min at room temperature. The filter paper was then carefully removed. A smear of band cells from cultures maintained for 24 or 48 h with or without sodium caseinate or rhG-CSF and the colonies on the coverslip were fixed and stained with Giemsa.

When the colonies contained small cells with ring-shaped nuclei or when the nuclei were segmented, they were scored as mature granulocytic colonies; when the cells were large in size and large vacuoles were evident, they were scored as macrophages. Finally, colonies with undifferentiated cells were scored as blastic.

Chromophore labeling of anti-M-CSF antibodies

The Atf-Bct-NHS labeling reagent (Boehringer, Mannheim) was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 1 mg/100 μL. A solution of 1 μg/mL of anti-M-CSF antibody was prepared, to which 1 μL of Atf-Bct-NHS was added per 9 μL of antibody solution. The entire process was performed under dimmed light. Antibodies and chromophore were incubated for 2 h at room temperature with constant shaking.

Detection of M-CSF using labeled anti-M-CSF antibodies

Neutrophils (3×10^6) from cultures treated with or without sodium caseinate or G-CSF were collected and washed twice in PBS. The cells were resuspended in 90 μL of RPMI, and 10 μL of chromophore-labeled antibodies were added and incubated in the dark for 20 min at 37 °C. Cells were washed twice in PBS to eliminate excess labeled antibodies; 100 μL of the cells were then used to prepare cytospin slides (30 μL per slide). The cells were fixed immediately with methanol, dried, and evaluated under a microscope using UV light (BH-2, Olympus, Japan).

ELISA assay

The primary antibody was goat-anti-mouse IgG anti-M-CSF and the secondary was a peroxidase-labeled rabbit-anti-goat antibody. An ELISA assay was used to detect M-CSF using the method described by Kemeny (Kemeny 1991). Briefly, 100 μL/well of conditioned medium or 12 ng of rmM-CSF in borate buffer (pH 9.5) was added to 96-well immunoplates (Costar, USA). After incubation for 2 h at 37 °C followed by incubation at 4 °C overnight, wells were washed with 0.1% (v/v) Tween 20 in PBS (Tween/PBS) and blocked by adding 100 μL/well 2% (w/v) BSA in PBS for 1 h at 37 °C. The BSA/PBS solution was discarded and 100 μL/well of the corresponding primary antibody (anti-M-CSF, goat anti-mouse IgG) were added, diluted to a concentration of 1:200 in BSA/PBS, and incubated for 3 h at 37 °C.

After washing with Tween/PBS, the secondary antibody (peroxidase-conjugated rabbit anti-goat) was added at a concentration of 1:3000 in BSA/PBS, and incubated for 2 h at 37 °C. Six milligrams of *ortho*-phenylendiamine were dissolved in 12 mL citrate buffer (pH 5) and used as a substrate for peroxidase; 10 μL of H_2O_2 were added to the solution immediately before use. A total of 100 μL/well were added and the plate was kept in darkness for 10 min at room temperature. The reaction was stopped by adding 25 μL/well of H_2SO_4 (2.5 N). The plates were read at 490 nm in an ELISA reader (Tecan Spectra, Austria).

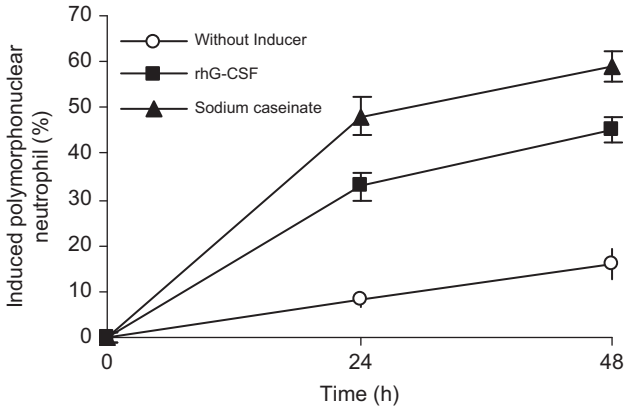

Fig. 1. Kinetics of differentiation of band cells from murine bone marrow towards polymorphonuclear neutrophils from 0 to 48 h of culture (RPMI + 10% FBS v/v) in 20 μL/mL sodium caseinate (10% in PBS), 20 ng/mL rhG-CSF or without inducer.

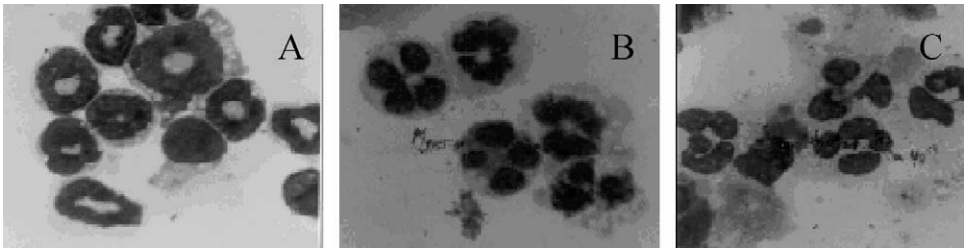

Fig. 2. Determination of cell morphology. Band BMCs cultured with or without 20 μL/mL sodium caseinate (10% in PBS) or 20 ng/mL rhG-CSF after 48 h of culture with RPMI-1640 medium and 10% FBS (v/v). (A) untreated cells; (B) treated with rhG-CSF; (C) treated with sodium caseinate. Magnification: × 400.

Inhibition of colony formation by anti-M-CSF antibodies

In order to confirm that the colony-forming activity of band cells treated with sodium caseinate or G-CSF was due to secretion of M-CSF, anti-M-CSF monoclonal antibodies were added to the colony assay (see above). BMCs were plated in agar and stimulated or not with the supernatants of band cells treated for 48 h with sodium caseinate or G-CSF. Anti-M-CSF antibodies were added to the first agar layer at a 1:200 dilution.

Results

Sodium caseinate induces differentiation of band cells into polymorphonuclear neutrophils

To determine whether sodium caseinate could stimulate the differentiation of neutrophils from band cells into polymorphonuclear cells, we cultured band cell-enriched murine bone marrow cells in the presence or absence of 20 μL/mL of sodium caseinate (10% in PBS), or 20 ng/mL rhG-CSF as a control, and the percentage of induced polymorphonuclear cells was evaluated after 24 and 48 h. After 24 h, sodium caseinate and rhG-CSF induced similar degrees of polymorphonuclear differentiation (48% and 33%, respectively, of the initial band cell population) (Fig. 1). In uninduced cultures, differentiation was seen in only 8% of the cells. The incidence of differentiation increased as a function of culture time, reaching 59% and 45% after 48 h of culture in the presence of either sodium caseinate or rhG-CSF (Figs. 1 and 2).

Neutrophils treated with sodium caseinate secrete a colony-stimulating factor

The colony-forming capacity of the supernatants and cell lysates of differentiated band cells was evaluated *in vitro*. For this purpose, 10^5 bone marrow cells were cultured in the presence of a 20% solution of the supernatants and lysates of band cells precultured for 24 and 48 h in the presence of sodium caseinate or rhG-CSF. A significant colony formation was observed

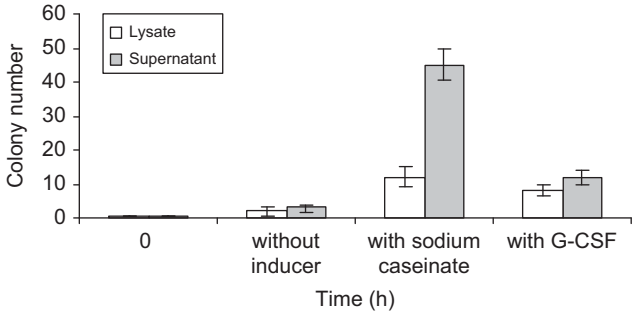

Fig. 3. Colony-forming assay. Lysate or supernatants of band neutrophils (10^6 cells/mL) treated or not with sodium caseinate or rhG-CSF for 48 h were collected and subsequently added (20% v/v) to 10^5 murine bone marrow cells/well in 24-well plates. The number of colonies was scored 7 days after plating.

Table 1. Cell morphology of colonies receiving either supernatants or lysates from band cells treated with sodium caseinate or rhG-CSF.

Inducer	Colony type (%)	
	Granulocytes	Macrophages
Without inducer	0 ± 0	0 ± 0
rhG-CSF	91 ± 2	9 ± 2
rmM-CSF	9 ± 3.5	91 ± 3.5
Lysates/sodium caseinate/48 h	0 ± 0	100 ± 0
Lysates/rhG-CSF/48 h	0 ± 0	100 ± 0
Supernatants/sodium caseinate/48 h	0 ± 0	100 ± 0
Supernatants/rhG-CSF/48 h	5 ± 2	95 ± 2

Morphology of cells from murine bone marrow colonies (10^5 cells/well) after 7 days of incubation in double layer colonies and stimulation with either supernatants or lysates (20% v/v) from band cells treated with sodium caseinate or rhG-CSF for 48 h. Colonies were collected via transfer onto coverslips and stained with Giemsa. Morphology was assessed using a microscope. 20 ng/mL rmMCSF and 20 ng/mL rhG-CSF were used as positive controls. rhG-CSF, recombinant human granulocyte colony-stimulating factor; rmM-CSF, recombinant murine macrophage colony-stimulating factor.

from supernatants and lysates obtained after 48 h and we found that this effect was particularly strong for supernatants from cells treated with sodium caseinate (Fig. 3).

As the identity of the factor with colony-forming activity was unknown, we evaluated the morphology of the cells forming the colonies. In assays using supernatants from band cells treated for 48 h with sodium caseinate, 100% of the cells in the colonies showed macrophage morphology, whereas with rhG-CSF treatment, 95% and 5% of cells showed macrophage and neutrophil morphology, respectively (Table 1). Bone marrow cells treated with band cell lysates only formed colonies containing cells with macrophage morphology. rhG-CSF and rmM-CSF were used as controls. The rhG-CSF promoted formation of granulocytes; 91% and 9% of the colonies were characterized as granulocytes and macrophages, respectively. When rmM-CSF was added to the cultures, 91% of the colonies were of macrophage lineage.

Band cells and polymorphonuclear cells produce M-CSF

We used a chromophore-labeled UV-sensitive anti-M-CSF antibody to detect M-CSF. The CaLo cervical carcinoma cell line, which does not express M-CSF (Fig. 4A), and L-929 cells that are known to produce M-CSF (Fig. 4B) were used as negative and positive controls, respectively. All granulocytic cells, regardless of treatment with rhG-CSF or sodium caseinate, were stained positively with the anti-M-CSF antibody (Fig. 4C–E). ELISA assay confirmed the presence of M-CSF in band cells cultured with or without sodium caseinate or rhG-CSF (Fig. 5).

M-CSF is the only factor responsible for colony formation by neutrophils cultured in the presence of sodium caseinate

A monoclonal anti-M-CSF antibody was used to assess whether the colony-forming activity obtained was due to M-CSF in the supernatants and lysates of cells treated with sodium caseinate. The antibody treatment suppressed colony formation (Fig. 6), suggesting that M-CSF is the only factor with colony-stimulating ability that is produced by neutrophils cultured in the presence of sodium caseinate.

Discussion

Granulocytes function as innate phagocytic and microbicidal cells in the immune system, and are important migratory cells in local tissue inflammatory responses (Appelberg 2007). It has long been known that whole casein, including sodium caseinate, is a potent inflammatory mediator that induces the chemotactic migration of neutrophils when injected into tissue (Metcalf et al. 1996). In this study, sodium caseinate induced the differentiation of band cells to segmented neutrophils to a similar extent as G-CSF (Figs. 1 and 2), a cytokine that mainly stimulates the proliferation and differentiation of granulocytes (Mora et al. 1992). Given that CasNa promotes granulocyte differentiation and inhibits the proliferation of several leukemic cell lines (Ramos-Mandujano et al. 2008), it could be interesting to determine whether it could also induce the differentiation of granulocytes from leukemic cells. If so, CasNa could be used to induce the differentiation of both normal and leukemic cells similar to *all-trans*-retinoic acid (ATRA), the first-line option for treatment of acute promyelocytic leukemia (Tallman et al. 2005).

The present study showed that sodium caseinate induces band cells to produce colony-forming activity (Fig. 3) of the macrophage colony type (Table 1). In fact, immunocytochemistry and ELISA assays showed that these cells produce M-CSF (Figs. 4 and 5). Antibody against rmM-CSF inhibited all the colony formation induced by the supernatants of these cells (Fig. 6), thus pointing that M-CSF is the only factor with colony-stimulating ability that is produced by neutrophils cultured in the presence of sodium caseinate.

Injection of sodium caseinate increases serum levels of colony-stimulating factors such as G-CSF, M-CSF or GM-CSF in the sera and exudates of the peritoneal cavities of mice (Lotem and Sachs 1985; Metcalf et al. 1996), but the identity of the cells producing these factors remains unknown. The present study showed that sodium caseinate induces bioactive macrophage colony-stimulating factor secretion from neutrophils (Fig. 6), suggesting that these cells might be responsible for increased levels of M-CSF in the serum and peritoneal cavity of mice after CasNa injection (Lotem and Sachs 1985). In addition, the induction of other hematopoietic growth factors by M-CSF (Fixe and Praloran 1997) suggests that leukopoiesis in casein-fed mice (Okano et al. 1991; Barceló et al. 1993) might result from M-CSF production by granulocytic cells, although the participation of other cytokines and other cells cannot be ruled out. Considering that macrophages are the main producers of G-CSF (Murata 2003) and also produce GM-CSF (Sallerfors 1994), it would be interesting to determine whether CasNa apart from G-CSF can also induce GM-CSF production as well as other interleukins such as interleukin-1 that is known to be secreted by macrophages after stimulation with b-casein (Wong et al. 1996).

Macrophages produce G-CSF, a chemoattractant for granulocytes (Papayannopoulou 2004), and in the present study we have shown that CasNa induces

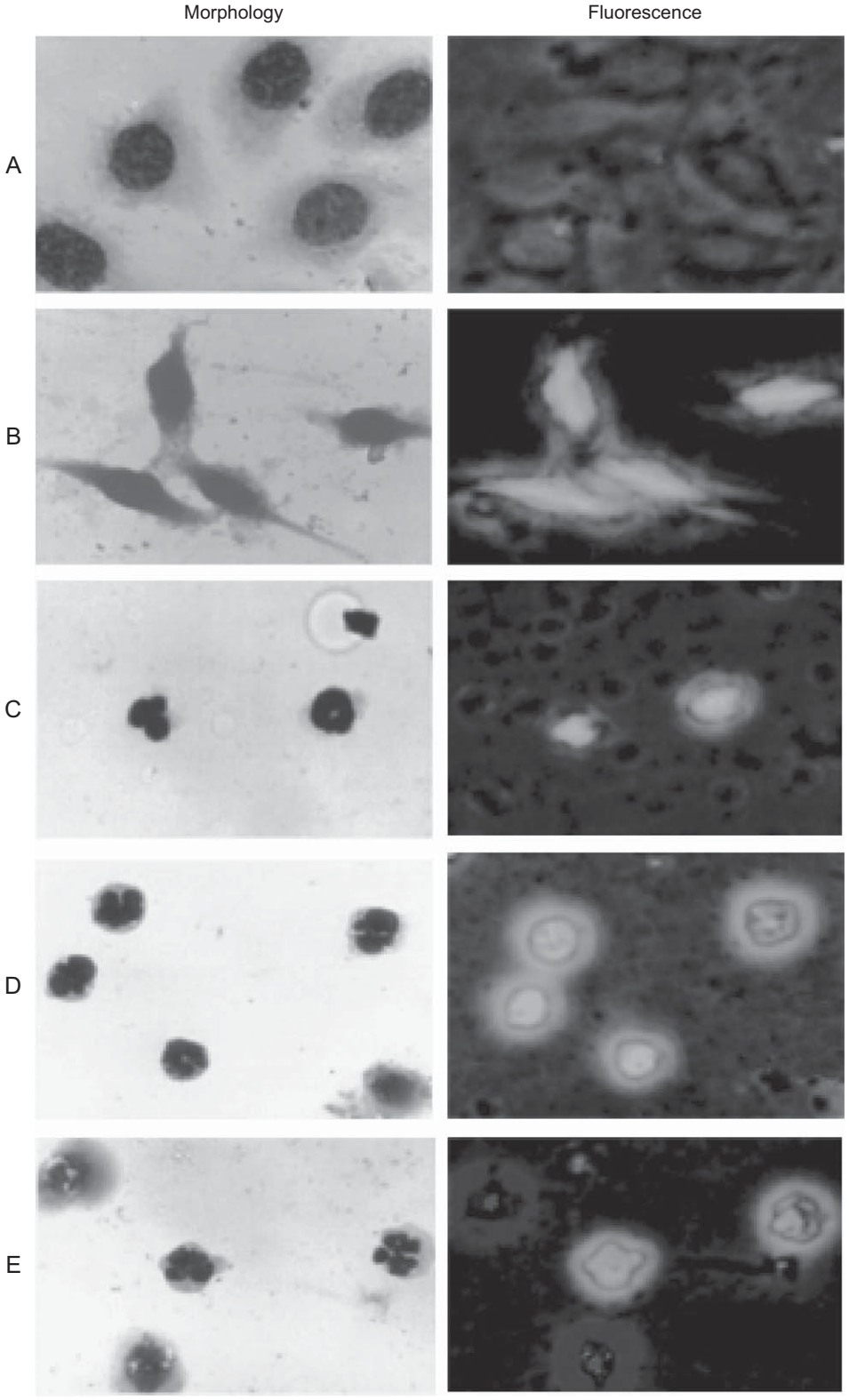

Fig. 4. Band cells cultured with and without sodium caseinate or rhG-CSF for 48 h were tested for their capacity to produce M-CSF. A chromophore-labeled (Atf-Bct-NHS label reagent) monoclonal anti-murine M-CSF antibody was used. After 48 h of culture, 3×10^6 band cells stimulated or not with sodium caseinate or rhG-CSF were washed twice with PBS, and resuspended in RPMI-1640 medium. Ten microliters of chromophore-labeled antibodies were added. After labeling, cytospin slides were prepared and cells fixed with methanol. On the right, UV light microscopy was employed, and on the left the same cells were stained with Giemsa (10% v/v) to reveal their morphology. (A) cervix carcinoma cell line (CaLo, negative control); (B) L-929 fibroblasts (positive control for M-CSF); (C) band cells cultured without inducer; (D) band cells cultured with rhG-CSF; (E) band cells cultured with sodium caseinate.

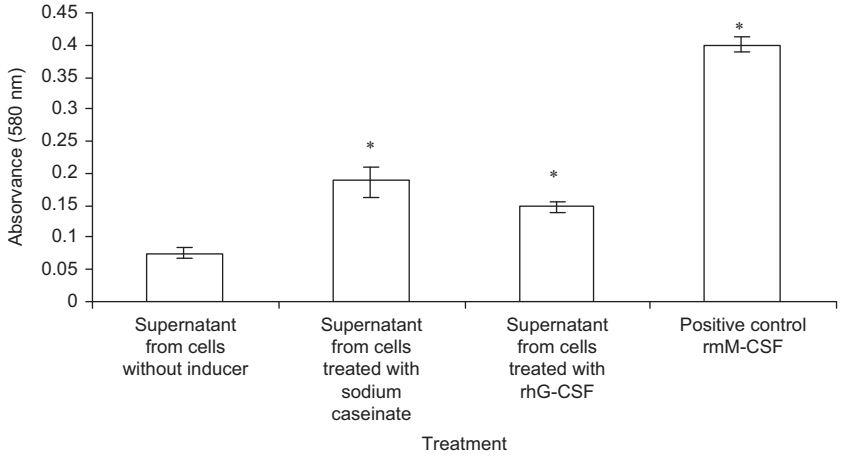

Fig. 5. M-CSF in supernatants of band cells treated with sodium caseinate or rhG-CSF for 48 h was identified by ELISA assay. The primary antibody was goat-anti-mouse IgG anti-M-CSF used at a dilution of 1:200 in BSA/PBS, and the secondary was peroxidase-labeled rabbit-anti-goat antibody (dilution of 1:3000 in BSA/PBS). Supernatants of band cells without treatment and rmM-CSF were used as negative and positive controls, respectively. *Results were significantly different ($p < 0.05$) from that of the control (0 mg/mL) as determined by Student's *t*-test.

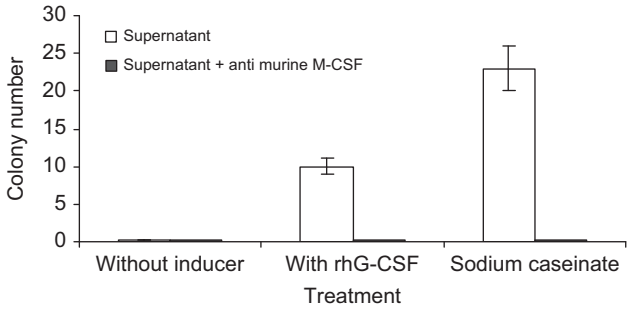

Fig. 6. Addition of murine anti-M-CSF antibody (1:200 dilution in the first agar layer) blocked colony-stimulating effect on BMCs (10^5 cell/well) of supernatants (20% v/v) from band cells treated for 48 h with or without sodium caseinate or rhG-CSF. No colonies formed in the absence of inducers when supernatants from band cells were added. Colony was inhibited by anti-M-CSF antibodies.

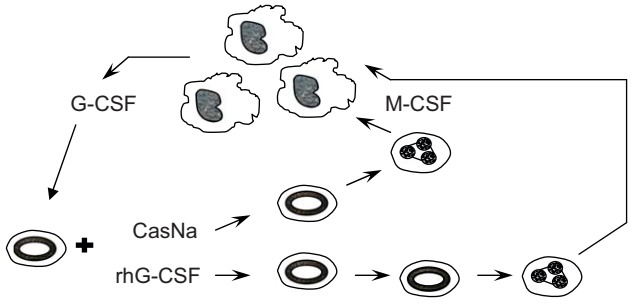

Fig. 7. Cytokine-mediated network communication between macrophage and neutrophil granulocytes populations. Macrophages produce G-CSF (granulocyte chemoattractant) and neutrophils produce M-CSF (macrophage chemoattractant) upon G-CSF or sodium caseinate stimulation. G-CSF, granulocyte-colony-stimulating factor; M-CSF, macrophage colony stimulating factor; CasNa, sodium caseinate.

granulocyte production of M-CSF, a well-known chemoattractant for macrophages (Dorsch et al. 1993). On the other hand it has already been shown that CasNa is also a chemoattractant for granulocytes (Lotem and Sachs 1985; Pasotti et al. 1993; Metcalf et al. 1996), then if we take into consideration that granulocytes are the first cells to arrive at inflammation sites (Appelberg 2007), the posterior arrival of macrophage could be due to the chemoattractant capacity of the M-CF produced by these cells, and then the differentiation of the already arrived granulocytes into polymorphonuclear active cells could be due to the secretion of G-CSF by the newly arrived macrophages; thus our data suggest the existence of a cellular communication network between the two cell populations through the production of M-CSF by granulocytes and G-CSF by macrophages (Fig. 7), allowing them to arrive at inflammatory sites and generate an important immunological barrier against pathogenic agents and probably tumor cells (Mapara and Sykes 2004; Holness et al. 1993). This hypothesis is supported by the casein-mediated protection of mice that have been inoculated with lethal doses of pathogenic bacteria (Noursadeghi et al. 2002), by the reduced risk of intestinal tumors in rats receiving casein-supplemented diet (Tatsuta et al. 1992), and by the reduced tooth decay in children who consume ice cream supplemented with CasNa (Shaw 1959). Taking all these data into consideration and our

results, it is possible that casein is not only involved in immunomodulation but that it can also modulate hematopoiesis, possibly via M-CSF production.

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