Available online at www.sciencedirect.com
ScienceDirect
Immunobiology 213 (2008) 133–141
Immunobiology
www.elsevier.de/imbio

Alpha-, beta- and kappa-caseins inhibit the proliferation of the myeloid cell lines 32D cl3 and WEHI-3 and exhibit different differentiation properties

G. Ramos-Mandujano[a,b], B. Weiss-Steider[a], B. Melo[a], Y. Córdova[a], E. Ledesma-Martínez[a], S. Bustos[a], O. Silvestre[a], I. Aguiñiga[a], N. Sosa[a], I. Martínez[a], L. Sánchez[a], A. García[a], E. Santiago-Osorio[a,*]

[a]*Laboratorio de Hematopoyesis y Leucemia, Facultad de Estudios Superiores Zaragoza, UNAM, Batalla 5 de mayo s/n, Col. E. Oriente, Iztapalapa, CP 09230, México, DF, México*
[b]*Departamento de Biofísica, Instituto de Fisiología Celular, UNAM, Ciudad Universitaria, CP 04510, México, DF, México*

Received 17 November 2006; received in revised form 22 June 2007; accepted 17 July 2007

Abstract

We have recently shown that sodium caseinate (CasNa) was able to inhibit the proliferation of the myeloid cell line 32D cl3 in a non-toxic way, and that it also induced the expression of macrophage colony-stimulating factor (M-CSF). Casein is the main protein present in milk and is composed of alpha (α), beta (β) and kappa (κ) subunits. This work was undertaken to evaluate if any one casein is responsible for the proliferation and differentiation properties found for CasNa on myeloid cells. Taking into consideration that 32D cl3 cells are considered to be non-malignant and dependent on IL-3 for proliferation, we also included for this study a leukemic cell line, WEHI-3, that does not depend on any external growth factor for its proliferation in order to evaluate if the growth inhibitory effect of caseins is also present for malignant cells. Our results showed that all caseins were inhibitory for the proliferation of either 32D cl3 and WEHI-3 and that only the 32D cl3 cells were induced to differentiate into the monocyte-macrophage lineage. In order to evaluate if CasNa was able to inhibit the proliferation of other myeloid cells we used J774 and P388 and found that they were also inhibited. We also determined that the different caseins exhibit different differentiation properties, with α-casein being the only one able to induce the secretion of M-CSF. We consider this work to open a new field of research, where casein, or its components, can be studied for their possible role in hematopoiesis and on the inhibition of malignant cell proliferation for therapeutic use.

Keywords: Casein; Differentiation. Hematopoiesis; Leukemia; Macrophage; M-CSF

Abbreviations: BMC, bone marrow cells; CasNa, sodium caseinate; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; Gr-1, Ly-6G granulocyte antigen specific; IL-3, interleukin-3; IMDM, Iscove's Modified Dulbecco's Medium; M-CSF, macrophage colony-stimulating factor; M-CSFR, macrophage colony-stimulating factor receptor; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase-polymerase chain reaction

*Corresponding author. Laboratorio de Hematopoyesis y Leucemia (L-8), UMIE, FES-Zaragoza campus II. Batalla 5 de mayo s/n, Col. Ejercito de Oriente, Iztapalapa, CP 09230, México, DF., México. Tel./fax: +52 55 5773 4108.
E-mail address: edelmiro@servidor.unam.mx (E. Santiago-Osorio).

0171-2985/$ - see front matter © 2007 Elsevier GmbH. All rights reserved.
doi:10.1016/j.imbio.2007.07.004

Introduction

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

Soon afterwards, the presence of several growth factors, like macrophage colony-stimulating factor (M-CSF), granulocyte-CSF (G-CSF) and granulocyte-macrophage-CSF (GM-CSF), were discovered in high concentrations in the sera of those mice treated with CasNa or casein (Lotem and Sachs, 1985; Metcalf et al., 1996). Actually, it was from these sera that the first hematopoietic growth factors were first discovered, purified and studied (Sachs, 1990). Now that recombinant growth factors are available (Kaushansky, 2006), inoculation with CasNa or casein has remained a good inducer of inflammatory cells in the peritoneal cavity of mice (Metcalf et al., 1996).

Our laboratory has recently shown that CasNa is an inhibitor of myeloid cell proliferation and a good inducer of their differentiation along the monocyte macrophage lineage (Ramos et al., 2000). In fact, 32D cl3 cells were inhibited in their *in vitro* growth in a dose-dependent way and the expression of mRNA for M-CSF was also induced (Ramos et al., 2004).

Casein is formed by alpha (α)-, beta (β)- and kappa (κ)-casein subunits (Meisel, 2005). A couple of those casein subunits have been shown to have several regulatory properties on lymphoid cells. For example, κ-casein can block splenocyte mitogenesis (Otani et al., 1992; Otani and Hata, 1995), while β-casein can help in the induction of proliferation of B and T lymphocytes (Wong et al., 1996); however, we do not know if some casein subunits can also modulate myelopoiesis.

This work was undertaken to evaluate the possible regulatory properties of the different caseins on cell proliferation and differentiation of myeloid cells. For this purpose, a mouse normal myeloid cell line dependent on interleukin-3 (IL-3) for its proliferation, 32D cl3, was used, as well as a leukemic myelomonocytic cell line, WEHI-3.

Materials and methods

Cytokines and antibodies

Recombinant mouse interleukin-3 (rmIL-3), recombinant mouse interferon gamma (rmIFN-γ), recombinant mouse macrophage colony-stimulating factor (rmM-CSF), anti-rmM-CSF purified goat IgG and a kit for M-CSF ELISA were purchased from R&D Systems (MN, USA). Anti-fms (M-CSFR extracellular domain), goat anti-rabbit IgG (H + L) fluorescein conjugate (Upstate, NY, USA) and FITC-conjugated rat anti-mouse Ly6G (Gr-1) were purchased from BD Biosciences Pharmingen (San Diego, CA, USA).

Cell culture

The mouse IL-3-dependent myeloid cell line 32D cl3 (Greenberger et al., 1983) was a kind gift from T. Hoang (Hemopoiesis and Leukemia Laboratory, Montreal, Quebec, Canada). The mouse myelomonocytic leukemia cell line WEHI-3, the macrophage leukemia cell line J774 and P388 were purchased from ATCC (VA, USA). The cells were cultured in a 5% CO_2 atmosphere at 37 °C, in Iscove's modified Dulbecco's medium (IMDM, Gibco BRL, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone, Utah, USA). Additionally, media for the 32D cl3 cell line was supplemented with 0.5 ng/mL of rmIL-3.

Cell proliferation assays

To evaluate cell proliferation, twenty thousand 32D cl3, J744, P388 or WEHI-3 cells were cultured for 48 h with or without CasNa (Difco Laboratories, USA) or α-, β- or κ-casein (Sigma, México) in 96-well plates (Costar, Cambridge, MA, USA). After this time, 0.5 μCi/ml of ^{3}H-thymidine (activity 185 mBq; Amersham Pharmacia Biotech, UK) was added, and cells were harvested 24 h later, washed once with PBS and lysed in NaOH (0.4 M). Finally, 2 mL of scintillation liquid was added and samples were analyzed in a Beckman beta-counter (Beckman LS6500, USA). The conditioned media (CM) collected at 72 h of culture without ^{3}H-thymidine were stored at −70 °C.

Cell viability

To determine the presence of necrotic cells, 32D cl3 or WEHI-3 cells were cultured for 72 h with or without CasNa or α-, β- or κ-casein in 96-well plates. 20 μL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (5 mg/mL saline) was added to each well and the samples were incubated for 4 h at 37 °C. The cells were lyzed and solubilized by the addition of 100 μL of 0.04 N HCl in isopropanol. The absorbance was determined at 590 nm with a microplate reader (Tecan Spectra, Austria).

Cellular differentiation

In order to identify different cellular lineages, specific cytochemistry stains were used, with α-naphthyl acetate

esterase for monocyte-macrophages and chloroacetate esterase for granulocyte-neutrophils (Li et al., 1973).

To assess further the differentiation of 32D cl3 and WEHI-3 cells in response to casein, expression of the macrophage cell surface marker c-fms (macrophage colony-stimulating factor receptor (M-CSFR)) (Anderson et al., 1982) and granulocyte Gr-1 (Hestdal et al., 1991) were examined using flow cytometry. Briefly, 5×10^5 cells were incubated with a specific antibody at a concentration of 10 mg/mL for 30 min at 4 °C and washed with fluorescence-activated cell sorter buffer (phosphate-buffered saline (PBS), 2% FCS, 0.1% BSA, 0.01% NaN_3) 2 times. Thereafter, cells were incubated with a fluorescein isothiocyanate (FITC)-labeled secondary antibody for 30 min. Fluorescence was analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) according to the standard procedures.

Assay for Fc receptors (FcR)

The Fc receptors (FcR) were evaluated by means of a variation of the EA rosette technique (Bianco et al., 1970). Briefly: anti-sheep red blood cells (SRBC) rabbit IgG (Sigma) was diluted 1:1600 in PBS and mixed with a non-agglutinating concentration of SRBC previously washed in PBS, and incubated at 37 °C for 30 min. The erythrocytes coated with antibody (EA) were washed 3 more times in PBS to remove free IgG and stored in PBS for a maximum of 5 days at 4 °C until used. 5×10^5 32D cells were mixed with EA at a concentration of 100 red cells for each white cell, adjusted to 1 mL and incubated at 37 °C for 30 min. Finally, the cellular pellet was gently dispersed with a pipette in cold IMDM and the white cells with more than 3 attached erythrocytes were considered as rosettes. A minimum of 300 cells were counted for every determination.

Colony-forming assay

To obtain bone marrow cells (BMCs) from female mice strain CD1, IMDM was flushed through individual mouse femoral shafts using a syringe. The cells from bone marrow were pooled, washed 3 times by centrifugation in IMDM medium and then resuspended in 2 mL of IMDM containing 10% (v/v) of FBS.

For the colony-forming assay, the double-agar layer technique was employed (Pluznik and Sachs, 1966). Briefly, a first layer with 0.6% (w/v) agar containing IMDM medium, 20% (v/v) horse serum (HS) and either 20 ng/mL rmM-CSF or 20% (v/v) of each CM to be tested was layered; a second layer with 0.3% (w/v) agar, IMDM medium and 10% HS (v/v) was overlayered with 1×10^5 BMCs per well. After 7 days of incubation, all the colonies with more than 20 cells were counted using an inverted microscope.

In order to inhibit colony-forming activity in the agar assay, an anti-rmM-CSF monoclonal antibody (1:200 dilution) was added in the first agar layer with the BMCs. We also used rmM-CSF (20 ng/mL) in the presence or absence of antibody as control.

ELISA assay

The presence of M-CSF in the supernatants from cultures of 32D cl3 cells treated with different caseins was measured using an ELISA assay according to the manufacturer's (Mouse M-CSF Immunoassay, R&D) instructions. The plates were read at 450 nm in a plate reader (Tecan Spectra, Austria).

RT-PCR mRNA assay for M-CSF and the M-CSF receptor

The 32D cl3 cells were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) for expression of M-CSF and the M-CSF receptor mRNAs. For this purpose, total RNA was isolated with Trizol (Trizol reagent, Invitrogen Life Technologies, CA, USA). Briefly, 32D cl3 cells (1×10^6) were cultivated in the presence or absence of 1 mg/mL CasNa or α-, β- or κ-casein. The cells were then washed with PBS with 1 mL of Trizol and 200 µl of chloroform added. The aqueous phase was collected and mixed with 500 µl isopropyl alcohol, and the precipitated RNA was placed in 15 µl of DEPC-treated H_2O. Subsequently, the RNA (1 µg) was reverse-transcribed (RT) with 25 U/mL of Moloney murine leukemia virus reverse transcriptase according to the manufacturer's instructions (Perkin-Elmer, GeneAmp RNA PCR N808-0017, New Jersey, USA). The RT was performed with incubations for 60 min at 42 °C and 10 min at 90 °C. The resulting complementary DNA (cDNA) was amplified by standard PCR (105 s at 95 °C, 35 cycles of 15 s at 95 °C and 30 s at 60 °C, 7 min at 72 °C) in a thermal cycler (Techne Model FGEN05TP, New Jersey, USA). PCR products were subsequently visualized by agarose gel electrophoresis (2%) containing ethidium bromide. The RT-PCRs were normalized for β-actin.

The primers used in the PCR for M-CSF were 5′-TCG ACA TGG CTG GGC TCC C-3′ and 5′-ATC AGG CTT GGT CAC CAC ATC-3′, while the primers for the M-CSF receptor were 5′-TTG TAC CGA GCT TGA AGA CCC-3′ and 5′-AGC GTT GAG ACT GAG AGC CC-3′; these primers amplify 527 bp and 552 bp fragments, respectively (Raivich et al., 1998). For β-actin, the primers 5′-GGG TCA GAA GGA TTC CTA TG-3′ and 5′-GGT CTC AAA CAT GAT CTG GG-3′ were used; these primers amplify a 238 bp fragment.

Data represent the mean ± SD of three independent experiments. Statistical significance was assessed using a

Student's *t*-test. All the biochemicals were from Sigma Chemical Co (St Louis, MO), unless otherwise specified.

Results

CasNa and α-, β- and κ-caseins differentially inhibit the proliferation of the myeloid cell lines 32D cl3 and WEHI-3

In order to evaluate if CasNa and its components, the α-, β- or κ-caseins, could inhibit the cellular proliferation of the 32D cl3 and WEHI-3 cell lines, cells were cultured in the presence of 0, 0.01, 0.1, 0.5 and 1.0 mg/mL of each molecule at a cell density of 2×10^4 and 3×10^3 cells per mL, respectively; rmIL-3 (0.5 ng/mL) was also routinely added to the 32D cl3 cell cultures to induce survival and proliferation.

Our results showed that CasNa was an inhibitor of 32D cl3 proliferation at a dose of 0.1 mg/mL (Table 1A). Interestingly, the caseins presented different inhibition characteristics. While the β-casein had almost no inhibition capability, the α- and κ-caseins were strong inhibitors, although less than CasNa, starting at 0.5 mg/mL. In contrast, CasNa was the molecule with less inhibitory capabilities on WEHI-3 cells than the other caseins (Table 1B), with κ-casein the most powerful one, presenting a strong inhibition even at 0.5 mg/mL. When 10 ng/mL of rmIFN-γ was used as a control for proliferation there was a 42±9.1% inhibition. Neither CasNa nor the caseins reduced cell viability below 90% at the doses employed (Table 2).

Table 1. Cell proliferation of 32D cl3 and WEHI-3 cell line treated with casein

mg/mL	Cell proliferation (%)			
	CasNa	α	β	κ
(A)				
0	100±10.5	100±7.9	100±6.1	100±7.2
0.01	101±11.2	93±11.6	94±5.7	102±9.0
0.1	51±25.0*	94±9.8	91±4.5*	101±14.2
0.5	36±23.0*	88±12.0*	92±8.1*	80±19.7*
1	22±20.0*	58±23.8*	82±10.9*	43±25.0*
(B)				
0	100±7.7	100±10.6	100±11.6	100±6.0
0.01	95±6.5	104±13.3	108±12.5	103±9.5
0.1	104±6.2	106±9.8	104±12.0	96±5.0
0.5	99±16.9	89±16.0	90±13.1	67±15.9*
1	86±9.0*	73±12.2*	71±11.6*	32±19.0*

Percentage of proliferation of 32D cl3 (A) and WEHI-3 (B) cells treated with either sodium caseinate (CasNa), α-casein (α), β-casein (β) or κ-casein (κ) after 3 days of incubation. The results are expressed as a mean±SD from cultures without casein. *Results are significantly different ($P<0.05$) from that of the control (0 mg/mL), as determined by Student's *t*-test.

Table 2. Cell viability of 32D cl3 and WEHI-3 cell line treated with casein

mg/mL	Cell viability (%)			
	CasNa	α	β	κ
(A)				
0	98±1.8	99±0.7	99±0.7	97±1.5
0.01	98±1.4	99±0.6	99±0.5	98±1.3
0.1	98±1.3	99±0.4	99±1.3	98±1.5
0.5	98±1.6	99±0.5	99±0.5	99±1.3
1	94±3.6	99±1.0	98±2.1	99±1.4
(B)				
0	98±1.4	99±1.1	98±0.3	99±0.1
0.01	98±1.2	98±0.9	98±0.3	98±0.1
0.1	98±1.6	98±1.2	98±0.2	98±0.3
0.5	98±1.9	98±1.0	97±0.4	94±1.5
1	97±0.9	96±1.5	92±1.5	89±2.6

Percent viability of 32D cl3 (A) and WEHI-3 (B) cell lines treated with sodium caseinate (CasNa), α-casein (α), β-casein (β) or κ-casein (κ) after 3 days of incubation, as determined by MTT assay. Results are expressed as a mean±SD from cultures without casein.

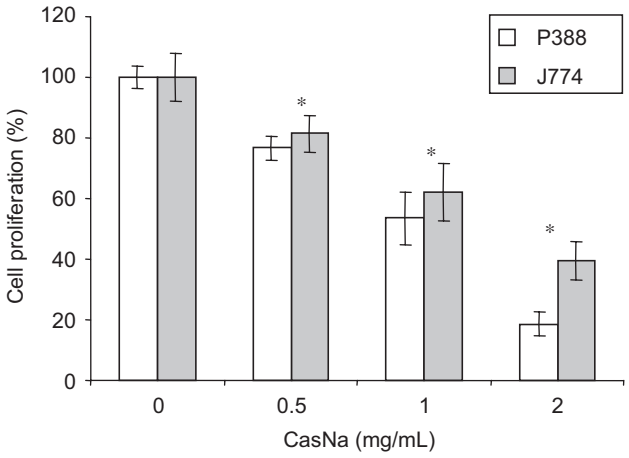

Fig. 1. Percentage of proliferation of P388 and J774 cells treated with 0, 0.5, 1 and 2 mg/mL of sodium caseinate (CasNa) after 3 days of incubation. The results are expressed as a mean±SD from cultures without casein. *Results are significantly different ($P<0.05$) from that of the control (0 mg/mL), as determined by Student's *t*-test.

In order to evaluate if CasNa was also capable of inhibiting the proliferation of other myeloid cells, we cultured the J744 and P388 cell lines in the presence of 0.5, 1 and 2 mg/mL of CasNa and found that they were significantly inhibited (Fig. 1).

In order to determine if the inhibitory effect of CasNa was in fact due to a protein, we treated the CasNa sample with trypsin for 30 min at 37 °C and found that the inhibitory activity was eliminated (Fig. 2). Thus we

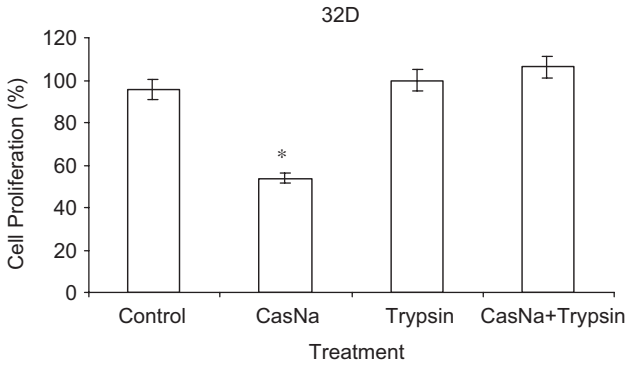

Fig. 2. Percentage of proliferation inhibition induced on 32D cl3 cells after 3 days of incubation with 1 mg/mL of CasNa that was autoclaved for 20 min at 120 °C and treated with (CasNa/Trypsin) and without (CasNa) 0.05% of trypsin (60 min at 37 °C). Controls with only IL-3 (Control) and IL-3 with trypsin (Trypsin) were also added. The results are expressed as a mean ± SD. *Results are significantly different ($P<0.05$) from that of the control as determined by Student's *t*-test.

can be fairly certain that the molecule with the inhibitory activity is in fact a protein.

On the other hand, taking into consideration that casein is the only protein in milk that does not denature at high temperatures (Swartz et al., 1992), we proceeded to autoclave the casein sample for 20 min at 120 °C and found that all the inhibitory activity was still present (Fig. 2). Finally, by using the specific property of casein that at high temperatures it is the only milk protein that forms aggregates (Lorient and Alais, 1970), we centrifuged the autoclaved sample at 9000*g* for 10 min in order to precipitate the fraction that was aggregated and proceeded to thoroughly wash away all the other molecules in suspension. Once again we found that the inhibitory activity was present in this precipitate. Thus we can be fairly sure that the casein is in fact the molecule responsible for the inhibitory activity we obtained on myeloid cell proliferation.

On the other hand, we also cultured the cells in the presence of 10–300 ng/mL of LPS from *Salmonella typhimurium* and found no inhibitory activity induced by this molecule.

CasNa and the α-, β- and κ-caseins induced cell differentiation towards the monocyte-macrophage lineage in 32D cl3 cells, while they could not induce differentiation in WEHI-3

Once the inhibitory capabilities of CasNa and the caseins in the proliferation of 32D cl3 and WEHI-3 were established, we proceeded to evaluate if they could also induce cell differentiation. For this purpose, we cultured the cell lines in the presence of 1 mg/mL of each casein under the same conditions as those used for cell inhibition. To evaluate the differentiation towards the macrophage lineage, we used the specific cytochemical stain for α-napthyl acetate esterase and also evaluated the expression of the membrane receptor for M-CSF (M-CSFR; also known as c-fms proto-oncogene), while for granulocytes, we used the cytochemical stain for chloroacetate esterase and the expression of the Ly-6G (Gr-1) membrane receptor.

Our results showed that CasNa and all the caseins were able to induce a strong differentiation to the macrophage lineage in 32D cl3 cells, while they could not induce any significant type of myeloid differentiation in WEHI-3 (Table 3A, B). It is interesting to note that, once again, there were different capabilities for induction of differentiation between CasNa and the caseins, with κ-casein being by far the weakest one (Table 3A). When 10 ng/mL of rmIFN-γ was used as a control for differentiation, a 33 ± 11.7% induction towards macrophage morphology was obtained. As far as the expression of cell receptors is concerned, CasNa and the α- and β-caseins induce M-CSFR, but CasNa was the best inducer for it (Table 3A). On the other hand, a small induction of the granulocyte lineage was also detected on 32D cl3, being more evident for the caseins than for CasNa (Table 3A).

Table 3. Cell differentiation of 32D cl3 and WEHI-3 cell line treated with casein

	Cytochemistry (%)		FACS	
	Granulocytes	Monocytes	(Gr-1)	(M-CSFR)
(A)				
Without inducer	51 ± 10.5	4 ± 1.9	1	1
CasNa (1 mg/mL)	26 ± 11.4	26 ± 4.1*	1.34	2.63
α (1 mg/mL)	34 ± 12.4	26 ± 11.7*	1.61	2.12
β (1 mg/mL)	40 ± 11.2	23 ± 8.7*	1.85	2.09
κ (1 mg/mL)	42 ± 9.8	13 ± 3.6*	1.5	1.31
(B)				
Without inducer	73 ± 10.1	3 ± 1.0	1	1
CasNa (1 mg/mL)	72 ± 11	2 ± 3.1	1.2	1.21
α (1 mg/mL)	66 ± 7.2	3 ± 1.2	1.34	1.47
β (1 mg/mL)	71 ± 10.3	4 ± 1.0	1.21	1.48
κ (1 mg/mL)	72 ± 8.1	3 ± 3.0	1.23	1.45

Percentage of granulocytes and macrophages obtained from 32D cl3 (A) and WEHI-3 (B) cell lines treated with sodium caseinate (CasNa), α-casein (α), β-casein (β) or κ-casein (κ) after 3 days of incubation and staining with alpha naphthyl acetate esterase, specific for macrophages, and chloroacetate esterase, specific for granulocytes. Results expressed as a mean ± SD from cultures without casein. The antibody anti-Gr-1, specific for granulocytes, and antibody anti-M-CSFR (c-fms), specific for macrophages, was detected by FACS and the result was expressed as the fold increase in fluorescence (treatment fluorescence/control fluorescence). *Results are significantly different ($P<0.05$) from that of the control (0 mg/mL), as determined by Student's *t*-test.

In order to evaluate if other differentiation properties were also induced by CasNa we measured the induction of FcR through a rosette assay: We found a significantly large induction of rosettes obtaining that $46 \pm 6.9\%$ of 32D cl3 cells formed rosettes when treated with 1 mg/mL of CasNa versus $10 \pm 1.5\%$ in untreated cells. When 10 ng/mL of rmIFN-γ was used as a control for FcR induction, $44 \pm 4.1\%$ of the cells formed rosettes.

CasNa and the caseins induce the expression of mRNA for M-CSF and M-CSFR in 32D cl3 cells and not in WEHI-3 cells, while only CasNa and the α-casein could induce the secretion of M-CSF in these cells

Once we determined that the caseins were able to induce protein expression of the receptor for M-CSF on 32D cl3 cells, we next evaluated if mRNA expression for M-CSF and M-CSFR was also induced in these cells, as well as in WEHI-3.

Using the same cell culture conditions as those for the differentiation assays, we determined that CasNa and all the caseins were able to induce mRNA expression for M-CSF and its receptor on 32D cl3 cells, but not in WEHI-3 cells (Figs. 3, 4A and B).

In order to evaluate if the caseins could induce M-CSF production in the cell lines, we measured by an ELISA assay the presence of M-CSF in the culture media. Interestingly, and once again pointing to the different inducing capabilities for the different caseins, we determined that only 32D cl3 cells were induced to release M-CSF by CasNa and the α-casein, with CasNa being the best inducer with 1.3 ng/mL, while the α-casein was somewhat lower with 0.3 ng/mL (Fig. 5).

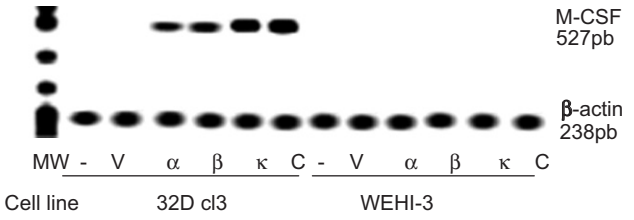

Fig. 3. Casein induces M-CSF mRNA expression in 32D cl3 cells but not in WEHI-3 cells. Both cell lines were cultured for 72 h with or without 1 mg/mL of different casein. Total RNA was extracted and RT-PCR was performed. The specificity of the cDNA was verified by the presence of products of the expected size on an agarose gel. MW, molecular weigh marker; – without inducer; V, vehicle; α, α-casein; β, β-casein; κ, κ-casein; C, sodium caseinate. β-Actin was used as a cellular control. The figure is a representative example of three different assays.

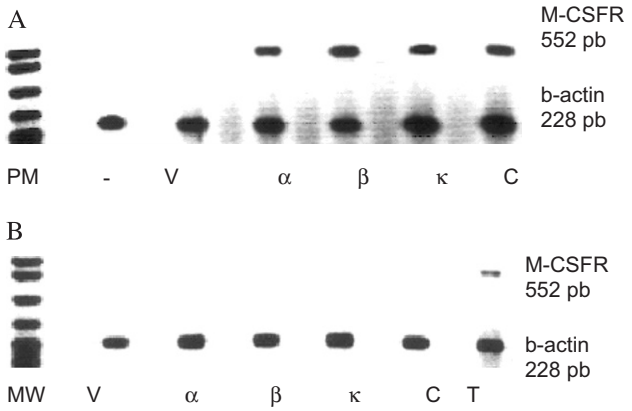

Fig. 4. Casein induces M-CSF receptor (M-CSFR) mRNA expression in 32D cl3 cells (A) but not in WEHI-3 cells (B). Both cell lines were cultured for 72 h with or without 1 mg/mL of different caseins. Total RNA was extracted and RT-PCR was performed. The specificity of the cDNA was verified by the presence of products of the expected size on an agarose gel. MW, molecular weigh marker; – without inducer; V, vehicle; α, α-casein; β, β-casein; κ, κ-casein; C, sodium caseinate. β-Actin was used as a cellular control. T, an additional assay from 32D cl3 cells treated with C was used as a control for M-CSFR RNA expression in WEHI-3 cells. The figure is a representative of three different assays.

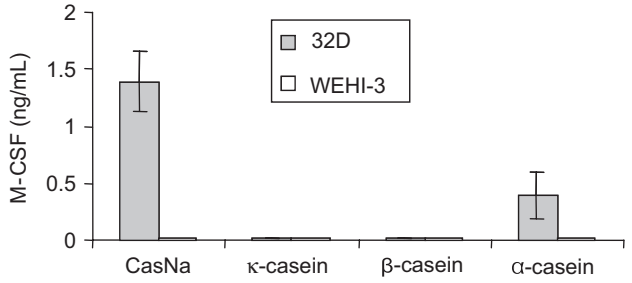

Fig. 5. CasNa and α-casein induce secretion of M-CSF in 32D cl3 cells. The conditioned media (CM) from 32D cl3 cells was collected after 48 h in culture in the presence of rmIL-3 (0.5 ng/mL) with 1 mg/mL of CasNa, α-, β- or κ-casein. The concentration (ng/mL) of M-CSF in CM was detected by ELISA assay.

The M-CSF secreted by 32D cl3 cells after induction by CasNa is bioactive and its specific antibody does not block the induction of its receptor on these cells

It is well known that M-CSF is a potent cell differentiator of myeloid cells towards the monocyte-macrophage lineage and that one of its important regulatory factors is the induction of other differentiation inducers and of their receptors, even the up-regulation of its own receptor (Gliniak and Rohrschneider, 1990). In order to evaluate if the M-CSF secreted by 32D cl3

cells through the induction of CasNa is bioactive, we used the conditioned media of 32D cl3 cells in a bone marrow colony forming assay. Our results showed that the M-CSF secreted by 32D cl3 cells under the induction of CasNa is bioactive, forming a similar number of colonies as those obtained with rmM-CSF, and that both were also completely inhibited by the use of anti-M-CSF (Fig. 6).

On the other hand, in order to evaluate if the presence of anti-M-CSF in these cultures could down-regulate the expression of the M-CSFR, we added anti-M-CSF to the culture media and evaluated the expression of the receptor for M-CSF in these cells. We determined that the presence of anti-M-CSF in the cultures did not down-regulate the expression of M-CSFR (Fig. 7).

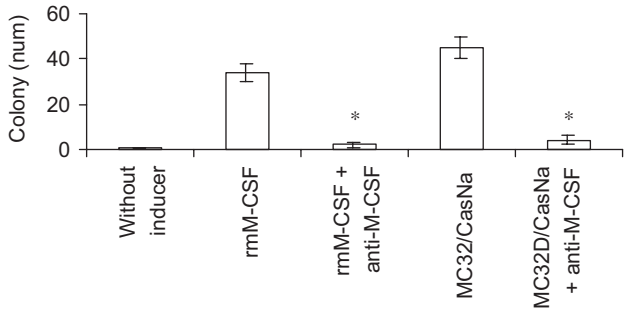

Fig. 6. The number of bone marrow colonies induced by CM of 32D cl3 cells cultured for 48 h with 0.5 ng/mL of rmIL-3 in presence of 2 mg/mL CasNa with or without anti-M-CSF (1:200 dilution). The number of colonies induced with 20 ng/mL of M-CSF in presence or absence of anti-M-CSF was evaluated as positive control. Agar cultures were kept for 7 days. The data are represented as the mean ± SD of three independent experiments and differences (*) were significant at $p < 0.05$, as determined by Student's *t*-test.

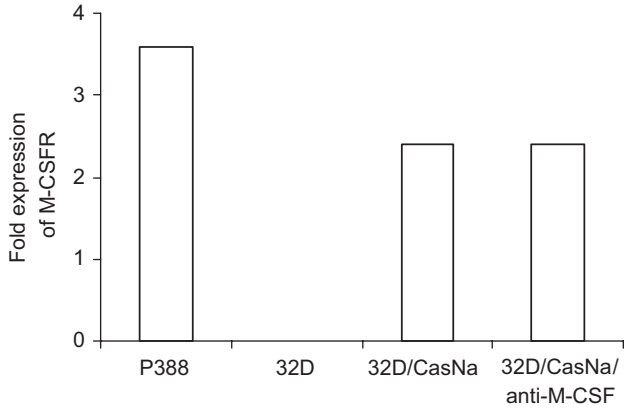

Fig. 7. Fold increase (treatment fluorescence/control fluorescence) over saline control of expression of M-CSFR antigen in 32D cl3 cells cultured for 72 h with CasNa in presence or absence of anti-M-CSF antibody. Expression of M-CSFR in the mouse macrophage cell line P388 was used as a positive control. 32D cl3, 32D cl3 cells without CasNa; 32D cl3/CasNa, 32D cl3 cells with CasNa; 32D cl3/CasNa/anti M-CSF, 32D cl3 cells with CasNa plus anti-M-CSF antibody.

Discussion

The mechanisms by which hematopoietic growth factors regulate blood cell proliferation and differentiation has been studied in detail and several interleukins and different types of endogenous cytokines have been described (Socolovsky et al., 1998; Kaushansky, 2006). Nevertheless, little is known of the existence of exogenous molecules that might have this effect. In this work we present evidence that milk caseins have the capacity to inhibit myeloid cell proliferation and to induce cell differentiation.

We determined that the α-, β- and κ-caseins have different potentials to regulate the proliferation and differentiation of two myeloid cell lines. The inhibition of cell proliferation and differentiation into the monocyte-macrophage lineage was obtained in 32D cl3 cells, while only cell inhibition and not differentiation was induced in WEHI-3 cells in a non-toxic way. It is interesting to note that CasNa, a salt of casein that contains all three caseins in its structure, was more effective in the inhibition of 32D cl3 cell proliferation but less effective in WEHI-3 cells; in fact, the κ-casein was in this case far more potent than the other caseins. These results point to the fact that the different caseins might have different hematopoietic properties.

It would be interesting to evaluate the inhibitory and differentiation properties of caseins in other myeloid cells in order to determine if this effect is characteristic of these cell types and to evaluate if the differentiation into macrophages can only be obtained in normal cells like 32D cl3 and not on leukemic cells like WEHI-3. Nevertheless, these data indicate that α-, β- and κ-caseins have myelopoietic properties, and the finding that these caseins could inhibit leukemic cell proliferation opens an interesting field of research to evaluate their possible therapeutic use.

It is interesting to note that even though all caseins were able to induce the expression of M-CSF in 32D cl3 cells, only the α-casein was able to induce its secretion. This result once again points to the different hematopoietic regulatory functions that the different caseins might have.

The caseins are known to contain in their structure potent bioactive peptides, some with opioid properties (Otani and Hata, 1995; Meisel, 2005). It would be thus interesting to evaluate if there are peptides in these molecules that might exhibit the different differentiation properties obtained in this work. Nevertheless, it is also interesting to note that receptors for complete casein molecules or CasNa have also been detected on myeloid and other cell types (Lewis and Van Epps, 1983; Hira

et al., 2003). Complete casein molecules, or bioactive peptides contained within their structure could have interesting blood cell regulatory properties that could open the way for a new field of basic and clinical research for this type of molecule.

Taking into consideration that the caseins induced in 32D cl3 cells the expression of mRNAs for M-CSF and for its membrane receptor, as well as other differentiation properties measured by specific cytochemical stains, and that CasNa also induced the secretion of bioactive M-CSF, we also evaluated the possibility that M-CSF was, in fact, the growth factor responsible for these differentiation properties. We determined that the induction took place even in the presence of anti-M-CSF, thus hinting at a different mechanism for macrophage induction of these cells. Published reports have demonstrated differentiation mechanisms unrelated to M-CSF for 32D cl3 cells towards macrophages due to the overexpression of inhibitory proteins of the CDK4 and CDK6 cycle (Adachi et al., 1997) or the activation of protein kinase C (Kovanen et al., 2000). Thus, it would be interesting to evaluate the possible role of the caseins in these types of regulatory mechanisms.

It has been published that caseins like κ-casein can inhibit lymphoid cell proliferation (Otani et al., 1992; Otani and Hata, 1995), while β-casein, on the contrary, can induce its proliferation (Wong et al., 1996; Monetini et al., 2003). These results and the ones presented in this work point to the multiple potential roles that caseins might have in hematopoiesis.

In conclusion α-, β- and κ-caseins, the components of casein, the main protein in milk, inhibit the proliferation of the normal and leukemic myeloid cell lines 32D cl3 and WEHI-3 in a dose- dependent way and present different differentiation properties. The 32D cl3 cells were induced to express mRNAs for M-CSF and M-CSFR, while CasNa and α-casein also induced the secretion of M-CSF, the specific growth factor for the monocyte-macrophage lineage.

Acknowledgments

We thank Mr. Ranulfo Pedraza Garnelo† for excellent technical assistance. This work was supported in part by PAPIIT (IN214903, IN217407) of the Universidad Nacional Autónoma de México (UNAM).

References

Adachi, M., Roussel, M., Haveith, K., Sherr, C., 1997. Features of macrophage differentiation induced by p19INK4d, a specific inhibitor of cyclin D-dependent kinases. Blood 90, 126–137.

Anderson, S.J., Furth, M.E., Wolff, L., Ruscetti, S.K., Sherr, C.J., 1982. Preparation of rat monoclonal antibodies to epitopes encoded by the viral oncogene (v-fms) of McDonough feline sarcoma virus. J. Cell. Biochem. 19, 275–280.

Bianco, C., Patrick, R., Nussenzweig, V., 1970. A population of lymphocytes bearing a membrane receptor for antigen–antibody complements complexes. I. Separation and characterization. J. Exp. Med. 132, 702–720.

Gliniak, B.C., Rohrschneider, L.R., 1990. Expression of the M-CSF receptor is controlled posttranscriptionally by the dominant actions of GM-CSF or multi-CSF. Cell 63, 1073–1083.

Greenberger, J.S., Sakakeeny, M.A., Humphries, R.K., Eaves, C.J., Eckner, R.J., 1983. Demonstration of permanent factor-dependent multipotential (erythroid/neutrophil/basophil) hematopoietic progenitor cell lines. Proc. Natl. Acad. Sci. USA 80, 2931–2935.

Hestdal, K., Ruscetti, F.W., Ihle, J.N., Jacobsen, S.E., Dubois, C.M., Kopp, W.C., Longo, D.L., Keller, J.R., 1991. Characterization and regulation of RB6-8C5 antigen expression on murine bone marrow cells. J. Immunol. 147, 22–28.

Hira, T., Hara, H., Tomita, F., Aoyama, Y., 2003. Casein binds to the cell membrane and induces intracellular calcium signals in the enteroendocrine cell: a brief communication. Exp. Biol. Med. 228, 850–854.

Kaushansky, K., 2006. Lineage-specific hematopoietic growth factors. N. Engl. J. Med. 354, 2034–2045.

Kovanen, P., Junttila, I., Takaluoma, K., Saharin, P., Valmu, L., Li, W., Silvenoinen, O., 2000. Regulation of Jak2 tyrosine kinase by protein kinase C during macrophage differentiation of IL-3-dependent myeloid progenitor cells. Blood 95, 1626–1632.

Lewis, S., Van Epps, E.D., 1983. Demonstration of specific receptors for fluoresceinated casein on human neutrophils and monocytes using flow cytometry. Inflammation 7, 363–375.

Li, C.Y., Lam, K.W., Yam, L.T., 1973. Esterases in human leukocytes. J. Histochem. Cytochem. 21, 1–12.

Lorient, D., Alais, C., 1970. Heat degradation of bovine α- and β-casein. I. Factors in variation of the degradation. Bull. Soc. Chim. Biol. 52, 915–926.

Lotem, J., Sachs, L., 1983. Control of in vivo differentiation of myeloid leukemic cells. III, regulation by T lymphocytes and inflammation. Int. J. Cancer 32, 781–791.

Lotem, J., Sachs, L., 1985. Independent regulation of myeloid cell growth and differentiation inducing proteins: in vivo regulation by compounds that induce inflammation. Int. J. Cancer 35, 93–100.

Meisel, H., 2005. Biochemical properties of peptides encrypted in bovine milk protein. Curr. Med. Chem. 12, 1905–1919.

Metcalf, D., Robb, L., Dunn, A., Mifsud, S., Rago, L., 1996. Role of granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor in the development of an acute neutrophil inflammatory response in mice. Blood 88, 3755–3764.

Monetini, L., Barone, F., Stefanini, L., Petrone, A., Walk, T., Jung, G., Thorpe, R., Pozzilli, P., Cavallo, M., 2003.

Establishment of T cell lines to bovine beta-casein and beta-casein-derived epitopes in patients with type 1 diabetes. J. Endocrinol. 176, 143–150.

Otani, H., Hata, I., 1995. Inhibition of proliferative responses of mouse spleen lymphocytes and rabbit Peyer's patch cells by bovine milk caseins and their digests. J. Dairy Res. 2, 339–348.

Otani, H., Monnai, M., Hosono, A., 1992. Bovine κ-casein as inhibitor of the proliferation of mouse splenocytes induced by lipopolysaccharide stimulation. Milchwissenschaft 47, 512–515.

Pluznik, D.H., Sachs, L., 1966. The induction of clones of normal mast cells by a substance from conditioned medium. Exp. Cell Res. 43, 553–563.

Ramos, G., Santiago, E., Martínez, I., Zambrano, I., Manrique, B., Weiss, B., 2000. El caseinato de sodio induce la diferenciación de las células hematopoyéticas multipotenciales 32D. Rev. Invest. Clin. 52, 638–644.

Ramos, G., Weiss, B., Córdova, Y., Hernández, J., Zambrano, I., Santiago, E., 2004. Sodium caseinate induces the murine multipotent myeloid cell line 32D to express and secrete the macrophage colony stimulating factor (M-CSF). Arch. Med. Res. 35, 109–113.

Raivich, G., Haas, S., Werner, A., Klein, M.A., Kloss, C., Kreutzberg, G.W., 1998. Regulation of MCSF receptors on microglia in the normal and injured mouse central nervous system: a quantitative immunofluorescence study using confocal laser microscopy. J. Comp. Neurol. 395, 342–358.

Sachs, L., 1990. The proteins that control haemopoiesis and leukaemia. Ciba Found. Symp. 148, 5–19 discussion 19–24.

Socolovsky, M., Constantinescu, S.N., Bergelson, S., Sirotkin, A., Lodish, H.F., 1998. Cytokines in hematopoiesis: specific and redundancy in receptor function. Adv. Protein Chem. 52, 141–198.

Swartz, M., Walker, N., Creamer, L., Southward, R., 1992. Casein and caseinates. In: Hui, Y.H. (Ed.), Encyclopedia of Food Science and Technology, vol. 1. Wiley-Interscience Publication, USA, pp. 310–317.

Wong, C.W., Seow, H.H.S., Liu, A.H., Husband, A.J., Smithers, G.W., Watson, D.L., 1996. Modulation of immune responses by bovine β-casein. Immunol. Cell Biol. 74, 323–329.