FULL PAPER *Immunology*

Generation of Multinucleated Giant Cells *In Vitro* from Bovine Monocytes and Macrophages

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(Received 29 October 2003/Accepted 15 April 2004)

ABSTRACT. The generation of multinucleated giant cells (MGC) from cells of the bovine monocyte-macrophage lineage was investigated. Freshly isolated monocytes were incubated with the conditioned medium (CM) of peripheral blood mononuclear cell cultures treated with Concanavalin A for 1–4 days (CM1 to CM4). Only CM1 generated MGC despite similar concentrations of IFNγ in all CMs. Nevertheless, MGC formation from monocytes was enhanced by adding either macrophage colony-stimulating factor (M-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF), MGC formations from macrophages were observed only when macrophages were cultured with GM-CSF plus CM. These results indicate that several mechanisms to generate MGC from bovine monocytes-macrophage lineage cells exist, and that GM-CSF is a major mediator of MGC formation in cattle.
KEY WORDS: cattle, GM-CSF, M-CSF, MGC.

J. Vet. Med. Sci. 66(9): 1065–1069, 2004

Multinucleated giant cells (MGC) are commonly observed in granulomas induced by the defence mechanism of the host to various stimuli. MGC formation is considered to originate in the fusion of monocyte-macrophage lineage cells. To clarify the mechanisms of MGC formation, several kinds of cytokines have been used to induce MGC from human monocytes or macrophages *in vitro* [1, 2, 4, 7, 8, 10, 13, 15]. In the field of veterinary medicine, *Mycobacterium avium paratuberculosis* is one of the most typical pathogens inducing granulomas in cattle and they proliferate in MGC [16]. Although several reports have described MGC in granulomas caused by infection with *M. avium paratuberculosis*, they have only been mentioned in histopathological studies [3, 9, 14], and the mechanism of their formation in granulomas remains unknown.

Macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are essential cytokines for the development, proliferation, and activity of monocyte-macrophage lineage cells. We therefore investigated the influence of M-CSF and GM-CSF on the generation of MGC *in vitro* from bovine monocytes and macrophages.

MATERIALS AND METHODS

Culture medium and reagents: RPMI 1640 medium (Invitrogen Corp., Carlsbad, CA, U.S.A.) supplemented with 10% heat-inactivated fetal calf serum (FCS; Intergen Company, Purchase, NY, U.S.A.), 100 U/m*l* penicillin, and 100 μg/m*l* streptomycin was used as a culture medium. Recombinant bovine M-CSF and GM-CSF were produced by baculoviruses carrying bovine M-CSF or GM-CSF cDNA [5,17]. Recombinant bovine IFNγ was generously provided by the Chuo-Sanken Laboratory of Katakura Industries (Sayama, Japan).

Conditioned media: Four kinds of conditioned media (CM) that differed in their cultivation time were prepared. The peripheral blood mononuclear cells (PBMC) were cultured in RPMI 1640 supplemented with 10% FCS and 10 μg/m*l* Concanavalin A (ConA) at a density of 1×10^7 cells/m*l* in 6-well plates for 1–4 days (Nunc, Roskilde, Denmark). The cell-free supernatants were collected as CM (CM1, CM2, CM3, and CM4) and stored at –20°C before use.

Preparation of CD14-positive monocytes: Monocytes were obtained by means of a magnetic cell separation system (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-bovine CD14 monoclonal antibody (mAb) (VMRD Inc., Pullman, WA, U.S.A.). Briefly, venous blood drawn from one 1-month-old Holstein, was diluted with an equal volume of phosphate-buffered saline (PBS), layered over Ficoll-Paque Plus (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and centrifuged at 400 × g for 30 min at 20°C. The PBMC band was removed and cells were washed twice with PBS. PBMC were suspended in RPMI 1640 medium containing 5% heat-inactivated fetal calf serum and incubated with anti-CD14 mAb at a 1:1,000 dilution for 60 min at 4°C. After washing, PBMC were incubated with anti-mouse IgG_1-coated microbeads (Miltenyi Biotec) for 15 min at 4°C, and CD14-positive cells were isolated by passing the PBMC through MACS with column-type LS+ (Miltenyi Biotec) according to the manufacturer's instructions.

Analysis of cell-surface antigens: Bovine macrophages induced from monocytes by recombinant bovine M-CSF or GM-CSF have not been fully characterized. Therefore we investigated the expression of cell-surface antigens of macrophages derived from monocytes. The CD14-positive cells were plated on 6-well cell culture plates (Sumilon, Tokyo,

Japan) at a density of 1×10^6/well with RPMI1640 supplemented with 10% FCS and either 100 *n*g/m*l* M-CSF or 10 *n*g/m*l* GM-CSF. After 5 days of culture, macrophages induced from monocytes were stimulated with recombinant bovine IFNγ (20 *n*g/m*l*) for 2 days, and harvested by incubation with PBS plus 5% FCS and 0.2% EDTA for 15 min. Macrophages were suspended in RPMI 1640 plus 5% FCS and 0.1% NaN_3 and incubated with one of the following: anti-bovine CD1, CD11b, CD14, MHC class I, MHC class II, or CD3 (VMRD Inc.). After exposure to the first antibody, the cells were washed with PBS and were incubated with rabbit anti-mouse IgG labeled with FITC (Cappel, Aurora, Ohio, U.S.A.). After washing, the cells were suspended in PBS plus 1% FCS and 0.1% NaN_3, and then analyzed with a flow cytometer (EPICS XL: Beckman Coulter, Inc., Miami, FL, U.S.A.).

Generation of MGC from freshly isolated monocytes: CD14-positive monocytes purified with MACS were cultured in RPMI 1640 supplemented with 10% FCS and a final concentration of 50% CM in 96-well tissue culture plates (Nunc, Roskilde, Denmark) at 1×10^5 cells per well. To investigate the influence of CSFs on MGC formation, either M-CSF (100 *n*g/m*l*) or GM-CSF (10 *n*g/m*l*) was added to the culture medium including CM. After 3 days of culture, the cells were removed from plates with 5 mM EDTA in PBS, stuck on slide glasses by Cytospin, and then stained with Diff Quick (Sysmex, Kobe, Japan). The fusion rate of monocytes was determined by counting the number of nuclei within MGC (≥ 3 nuclei/cells), and the fusion index was calculated according to the following formula: (number of nuclei within MGC)/(total number of nuclei counted) × 100.

Generation of MGC from macrophages induced by CSFs: To induce control macrophages, CD14-positive monocytes were cultured in RPMI 1640 supplemented with 10% FCS for 5 days (C-MΦ). To generate macrophages induced by CSFs, monocytes were cultured in 10% FCS RPMI 1640 supplemented with either 100 *n*g/m*l* M-CSF or 10 *n*g/m*l* GM-CSF for 5 days (M-MΦ or GM-MΦ, respectively). After the supernatants were removed from each macrophage culture, macrophages were cultured with 10% FCS RPMI 1640 at a final concentration of 50% CM1 supplemented with either 100 *n*g/m*l* M-CSF or 10 *n*g/m*l* GM-CSF for 3 days. The fusion index of macrophages was determined by the method described above.

Phagocytosis of latex particles: To investigate phagocytosis of MGC, 10 μl of latex particles (diameter of 2 μm; Polysciences, Inc. Warrington, PA, U.S.A.) adjusted to 1×10^9 particles/m*l* was added to MGC cultures induced by GM-CSF and CM1. Three hours later, the supernatants were removed, and after washing the surface of the plates with PBS, the cells were removed from the plates with 5 mM EDTA in PBS, and stuck on slide glasses with Cytospin, and then stained with Diff Quick. Latex particles taken into MGC were observed under a microscope.

Concentrations of bovine IFN-γ in CMs: Bovine IFN-γ was measured by an anti-bovine IFN-γ ELISA as reported by Jungersen *et al.*, but with slight modifications [6]. Briefly, 96-well plates (MaxiSorp, Nunc) were coated overnight with an anti-bovine IFN-γ monoclonal antibody (clone cc302, Serotec, Oxford, U.K.). Samples diluted with 1% skim milk in TBS-0.05% Tween20 were added to the coated plates, incubated for 2 hr at room temperature, and incubated with biotin-labeled anti-recombinant bovine IFN-γ rabbit IgG and subsequently with streptavidin horseradish peroxidase-conjugates (ELISA grade, Biosource, Camarillo, Cal, U.S.A.). After incubation with peroxidase substrate (TMB Microwell Peroxidase Substrate System, KPL, MD, U.S.A.), optical density at 450 nm was measured, and concentrations of IFN-γ were calculated according to the dose-response curve obtained from ELISA with recombinant bovine IFN-γ [12].

RESULTS

The concentrations of IFN-γ among these CMs were very similar, approximately 5 *n*g/m*l* (5.2–5.4 *n*g/m*l*), and were not detected in culture medium (Data not shown).

The figures of freshly isolated CD14-positive monocytes and each macrophage are shown in Fig. 1. Although a few neutrophil-like cells were observed in the freshly isolated CD14-positive monocytes, all the cells obtained after cultivation for five days were morphologically macrophages. M-MΦ and GM- MΦ had larger amounts of cytoplasm than C-MΦ.

The expression of CD1, CD11b, CD14 and MHC class I and II antigens was observed on both M-MΦ and GM-MΦ. The expression levels of MHC class I and II were increased by stimulation with IFNγ in both macrophages. The two kinds of macrophages showed the same expression pattern, except that the expression of CD14 in M-MΦ was slightly higher than that of GM-MΦ. Because all markers were expressed as one population, these macrophages had very similar characteristics with regard to cell surface antigens investigated (Fig. 2).

A typical MGC is shown in Fig. 3-A. This MGC was observed in the culture of monocytes with GM-CSF plus CM1. Figure 3-B shows that MGC has the ability to take up latex particles.

The fusion index of freshly isolated CD14-positive monocytes cultured with CM in the presence or absence of CSFs is shown in Fig. 4. When monocytes were incubated with CM in the absence of other added CSFs, only CM1 could generate MGC (9.1 ± 4.7%). When monocytes were incubated with CSFs in the absence of CM, only GM-CSF could generate MGC (4.5 ± 2.5%). Although the combination of CM and CSFs enhanced MGC formation, GM-CSF was more effective than M-CSF.

The fusion index of macrophages induced from monocytes is shown in Fig. 5. MGC formation of GM-MΦ was promoted in all cultivation conditions. In contrast, MGC formation was not enhanced in either M- MΦ or C- MΦ except during incubation with GM-CSF plus CM1.

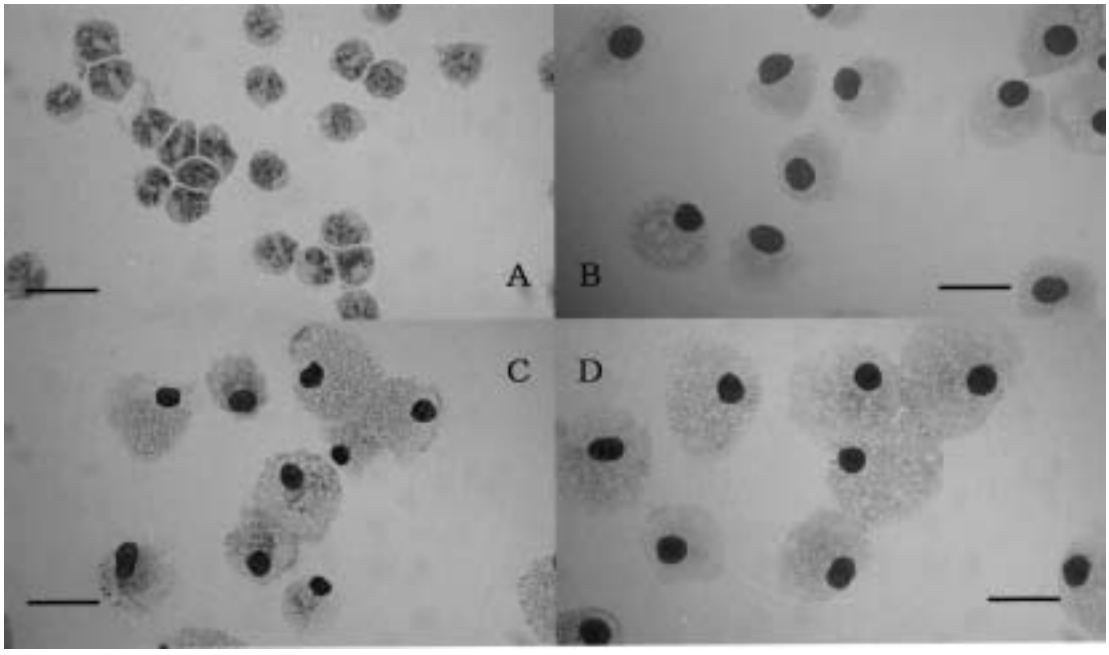

Fig. 1. Figure of CD14-positive monocytes (A), C-MΦ (B), GM- MΦ (C), and M- MΦ (D). Cells were stuck on slide glasses with Cytospin (900 rpm, 3 min), and stained with Diff Quick. The bar is 30 μm.

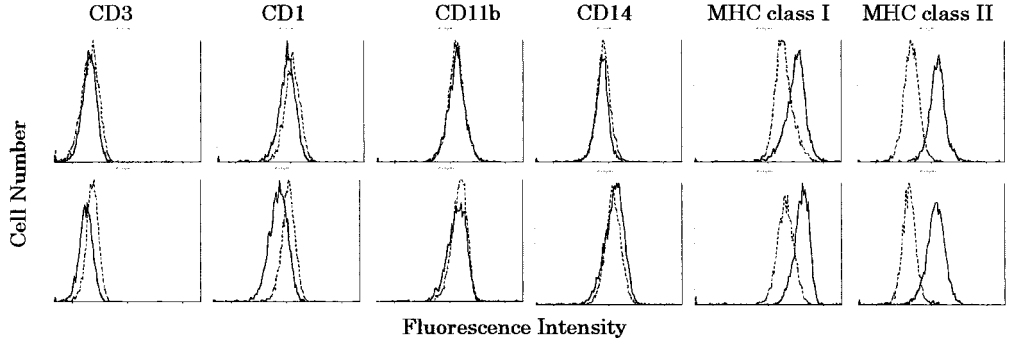

Fig. 2. Expression of cell surface antigens in GM- MΦ and M- MΦ. Monocytes were incubated with either GM-CSF or M- CSF. After 5 days of culture, macrophages induced from monocytes were incubated with or without IFNγ for 2 days. Dotted line: without IFNγ stimulation; Solid line: with IFNγ stimulation. Upper: GM-MΦ, Lower: M-MΦ.

DISCUSSION

Generation of MGC from freshly isolated monocytes was enhanced by incubation with CM plus either GM-CSF or M-CSF, whereas the generation of MGC in cultures of macrophages was observed only in the medium of CM plus GM-CSF. These results suggest that several mechanisms of the fusion process to generate MGC exist in monocytes, and that the effect of M-CSF disappears with the maturation of monocytes into macrophages. It has been reported that the ability of monocytes to form MGC is gradually lost *in vitro* during maturation into macrophages [11]. Because freshly isolated monocytes were cultured for 5 days to transform macrophages and continuously for 3 days to investigate the formation of MGC, the total incubation time was 8 days. When macrophages were cultured with M-CSF for more than 1 month, MGC formation was observed (data not shown). Because the results in this study were obtained from an 8-day culture of macrophages, the culture period may have been too short to generate MGC by stimulation of M-CSF.

GM-CSF generated MGC from freshly isolated monocytes without CM (Fig. 4), but in the case of macrophages, GM-CSF required CM to generate MGC (Fig. 5). Abe *et al.* have reported that GM-CSF is a major mouse alveolar macrophage fusion factor in CM [1]. McNally and Anderson

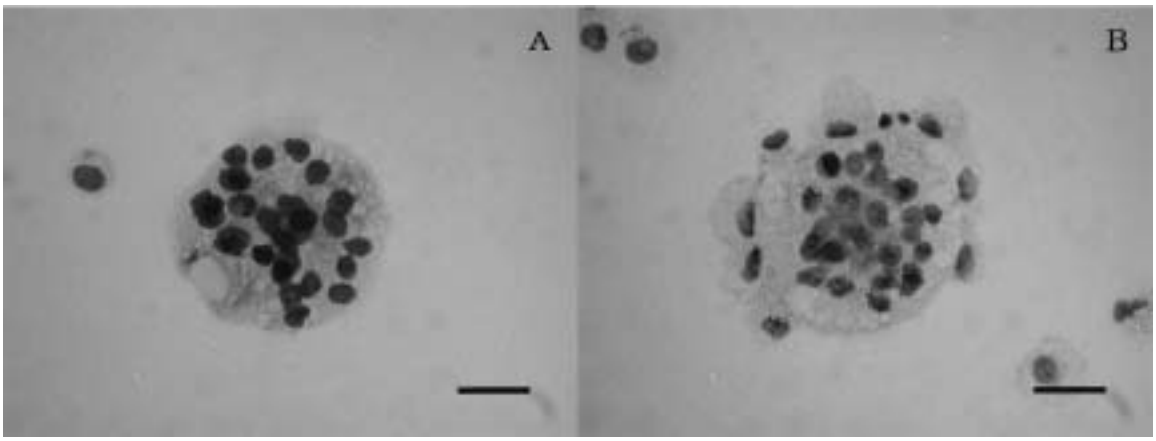

Fig. 3. Figure of MGC. (A) A typical MGC generated by CM1 plus GM-CSF. (B) MGC showed the ability to take up latex particles. The bar is 20 μm.

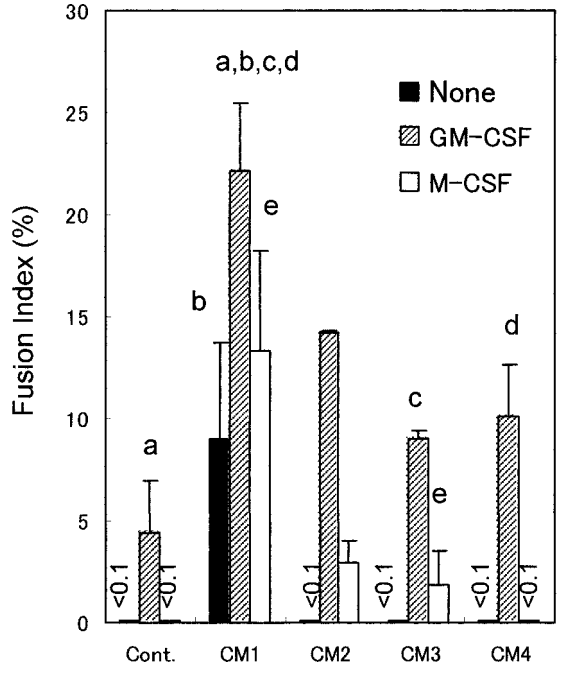

Fig. 4. MGC formation from bovine monocytes cultured in different CMs (CM1-CM4), or without CM (Cont.). CD14-positive monocytes were cultured in RPMI 1640 supplemented with 10% FCS and a final concentration of 50% CM in 96-well tissue culture plates at 1×10^5 cells per well for 3 days (solid bar). GM-CSF (hatched bar) or M-CSF (open bar) was added at the beginning of the culture. Data show mean values ± standard errors of the means. (n=2) Significant differences are indicated by the same alphabet letters ($p<0.05$).

have described how GM-CSF enhances the formation of MGC from human monocyte-derived macrophages by IL-4 [7]. Taking these reports together with our present findings, even though other essential factors are required, GM-CSF plays a crucial role in MGC formation from macrophages in cattle.

Several reports have described how IL-4 enhances the generation of MGC [1,2,7], but the fusion rate of mouse alveolar macrophages incubated with 10 *n*g/m*l* of GM-CSF was 30.1 ± 5.3%, and adding 4 *n*g/m*l* of IL-4 together with 10 *n*g/m*l* of GM-CSF increased the fusion rate to 94.5 ± 1.2% [1]. The highest fusion rate in our study was 22.2 ± 3.3% when monocytes were cultured with GM-CSF plus CM1. Further studies on the role of IL-4 are needed to clarify the precise mechanism of MGC formation in cattle.

Many reports have indicated that IFN-γ is the essential factor in CM promoting monocyte fusion [4, 10, 15]. We prepared four kinds of CMs that were different in incubation time during ConA stimulation. Although the concentrations of IFN-γ among these CMs were almost the same (approximately 5 *n*g/m*l*), MGC formation could hardly be obtained with freshly isolated monocytes, except CM1. The results of the present study do not allow us to deny the necessity of IFN-γ in MGC formation, but one or more essential factors other than IFNγ must be present in CMs.

We have reported herein the generation of MGC *in vitro* by culturing bovine monocytes and macrophages with either GM-CSF or M-CSF in combination with CM. Whereas MGC formations from macrophages were observed when macrophages were cultured with GM-CSF plus CM, MGC formations from monocytes were observed in various conditions. Monocytes gathering in an inflammation site should fuse together in a microenvironment where GM-CSF and other factors exist, and differentiate into MGC with phagocytic ability.

ACKNOWLEDGEMENT. This work was supported by a Grant-in-aid from the Recombinant Cytokine Project (PCR2002–2110–2), provided by the Ministry of Agriculture, Forestry and Fisheries.

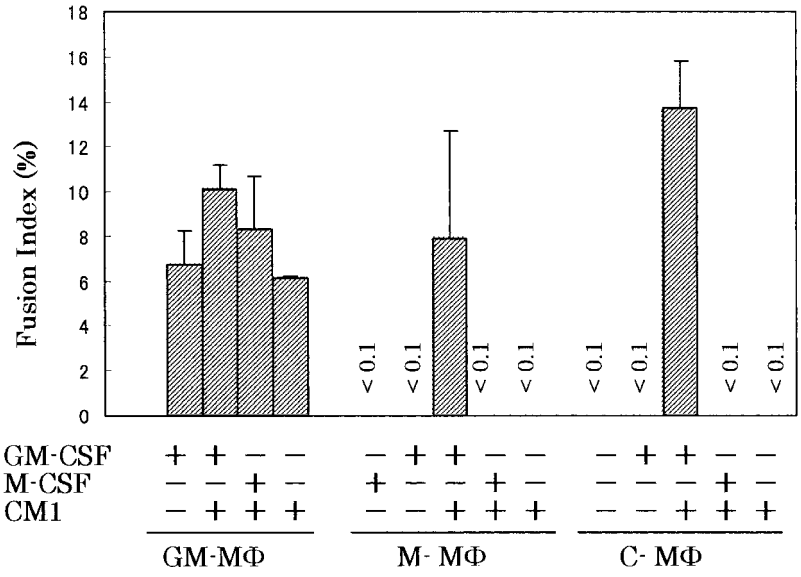

Fig. 5. MGC formation from macrophages. Macrophages were induced from freshly isolated CD14-positive monocytes by cultivation with GM-CSF (GM- MΦ), M-CSF (M- MΦ), or without CSF (C-MΦ) for 5 days. Each macrophage was incubated with a combination of GM-CSF, M-CSF, and CM1.

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