

Potentialiation by human serum of anti-inflammatory cytokine production by human macrophages in response to apoptotic cells

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Abstract: Phagocytosis of apoptotic cells by macrophages leads to the production of anti-inflammatory cytokines, thereby preventing inflammation. In this study, we demonstrate that human serum potentiates the production of anti-inflammatory cytokines, IL-10 and TGF- β , by PMA-treated THP-1 cells and human monocyte-derived macrophages in response to apoptotic cells, which results in great suppression of the production of proinflammatory cytokine IL-8. Human IgG but not its F(ab)'₂ suppressed the IL-8 production. Pretreatment of macrophages but not apoptotic cells with human serum or human IgG caused the suppression, suggesting that immune complex may not be formed with apoptotic cells. When Fc γ RI was specifically down-modulated by a monoclonal antibody, M22, the potentiating effects of human serum and human IgG on the anti-inflammatory cytokine production and the suppressive effects on IL-8 production were completely abolished. Thus, human IgG and Fc γ RI appear to be critical in leading to the production of anti-inflammatory cytokines by macrophage in response to apoptotic cells. *J. Leukoc. Biol.* 71: 950–956; 2002.

Key Words: IgG · Fc γ RI · IL-10 · TGF- β · phagocytosis

INTRODUCTION

It is generally believed that apoptotic cells are phagocytosed by neighboring phagocytes, such as macrophages, without inflammation [1]. There are two possible mechanism(s) through which inflammation can be avoided: one is no production of proinflammatory mediators, and the other is production of anti-inflammatory mediators, although these two possibilities are not mutually exclusive. Indeed, human monocyte-derived macrophages produced almost no proinflammatory cytokines such as interleukin (IL)-8 in response to apoptotic cells [2]. Subsequently, lipopolysaccharide (LPS)-induced IL-10 production of human peripheral blood monocytes reportedly was enhanced by coculturing with apoptotic cells [3]. Moreover, coculturing human monocyte-derived macrophages with apoptotic cells was also shown to produce anti-inflammatory mediators such as transforming growth factor- β (TGF- β) and prostaglandin E₂, by which inflammatory cytokine production was suppressed

[4]. Thus, the production of anti-inflammatory cytokines is now believed to be a key element for noninflammatory clearance of apoptotic cells [5, 6], which must be controlled by various means. However, what can down- or up-modulate the anti-inflammatory cytokine production remains largely unknown.

In this study, we demonstrate that human serum (HS) potentiates anti-inflammatory cytokine production by a phorbol 12-myristate 13-acetate (PMA)-treated human monocytic cell line, THP-1 cells, and human monocyte-derived macrophages in response to a variety of apoptotic cells, by which proinflammatory cytokine production is greatly suppressed. Moreover, we demonstrate that human immunoglobulin G (hIgG) appears to bind to macrophages, but not apoptotic cells, through the Fc receptor for IgG (Fc γ R)I to potentiate anti-inflammatory cytokine production.

MATERIALS AND METHODS

Preparation of cells and phagocytosis

THP-1 cells were maintained in RPMI-1640 medium containing 10% fetal calf serum (FCS; Gibco-BRL, Grand Island, NY). The cells were washed with phosphate-buffered saline (PBS) three times, followed by incubation in RPMI-1640 medium containing 10% FCS and 160 nM PMA for 72 h at 37°C at a cell density of 5×10^5 cells/ml. Human monocyte-derived macrophages were obtained by culturing plastic-adherent human peripheral blood mononuclear cells in RPMI-1640 medium containing 10% FCS and 1000 U/ml human macrophage colony stimulating factor (M-CSF) derived from human urine (Denka, Tokyo, Japan) for 3 days, followed by replacement of one-half of the medium with the fresh medium containing M-CSF and then culturing the cells for an additional 4 days. Cytolytic T lymphocyte cell line, CTLL-2 cells, was maintained in RPMI-1640 medium containing 10% FCS, 5×10^{-5} M 2-mercaptoethanol, and 100 U/ml recombinant human IL-2 (kindly provided by Takeda Pharmaceutical Co., Osaka, Japan). To induce apoptosis, CTLL-2 cells were washed with PBS three times, followed by incubation in an IL-2-free medium for 28 h at 37°C at a cell density of 5×10^5 cells/ml unless otherwise stated. The apoptotic cells thus obtained were positive for propidium iodide (PI) and fluorescein isothiocyanate (FITC)-Annexin V, but their membranes appeared to be intact because they did not release any lactate dehydrogenase, a cytosolic enzyme [7]. Human neutrophils were obtained from peripheral blood with a Ficoll density gradient centrifugation followed by hemolysis. The purity of the cells was 99%. To induce apoptosis, neutrophils were cultured in RPMI-1640 medium containing 10% autologous serum for 24 h at 37°C at a

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cell density of 5×10^6 cells/ml. Apoptosis occurred in less than 50% of total population, as assessed by FITC-Annexin V, and at this stage, all the cells were viable (trypan blue-negative, PI-negative). An in vitro-derived line from murine methylcholanthrene-induced neoplasm, P388 cells, was adjusted to the cell density of 10^6 cells/ml in the culture medium, followed by the addition of 1 mg/ml etoposide (WAKO, Osaka, Japan) to the final concentration of 1 μ g/ml. Then the cells were incubated for 5 h at 37°C. The apoptotic cells thus obtained were positive for FITC-Annexin V but not PI. To induce necrosis, CTLL-2 or P388 cells were washed with prechilled PBS three times, followed by adjustment of the cell density to 10^6 cells/ml in PBS. The cells were then subjected to freezing at -80°C for 30 min and thawing at 37°C for 5 min. B10.Thy 1.1 mice (8 weeks, female) were irradiated with X-rays (4 Gy) from a Shimai-III X-ray generator (Shimazu Seisakusho Ltd., Kyoto, Japan) at the National Institute of Radiological Sciences, and 3 h after irradiation, thymocytes were obtained as apoptotic cells. As we found previously, early apoptosis (PI-negative, Annexin V-positive) was notably induced under these conditions [8]. PMA-treated THP-1 cells or monocyte-derived macrophages were washed with PBS three times, followed by the addition of twofold excess number of apoptotic cells and incubation for 3 h at 37°C in RPMI-1640 medium containing 10% FCS, 10% HS (Nippon Seiyaku, Tokyo, Japan), 5 mg/ml hIgG (Nippon Seiyaku), or 5 mg/ml F(ab)'₂ of hIgG (Nippon Seiyaku).

Flow cytometric analysis

IL-2-deprived CTLL-2 cells were stained with PI, followed by incubation with PMA-treated THP-1 cells for 3 h at 37°C. The cells were then washed with PBS three times and placed on ice. Adherent cells were recovered by gentle flushing with prechilled PBS and tapping of the bottom of the plate. The cell size and PI fluorescence were analyzed by flow cytometry using FACScan and Cell Quest software (Becton Dickinson, Mountain View, CA). The expression of Fc γ RI and Fc γ RII was analyzed with PE-conjugated mouse anti-human CD64 (Fc γ RI) antibodies (clone 10.1, IgG1; Caltag Laboratories, Burlingame, CA) and PE-conjugated mouse anti-human CD32 (Fc γ RII) antibodies (clone 2E1, IgG2a; Beckman Coulter, Tokyo, Japan).

Measurement of cytokines

After coculturing, the cells were centrifuged, and the supernatants were harvested. Samples were stored at -80°C until the assay. The human IL-8 level was determined by means of a specific enzyme-linked immunosorbent assay (ELISA). The reagents were provided by Dr. K. Matsushima (University of Tokyo School of Medicine, Japan), as described previously [9], except that polyclonal rabbit anti-human IL-8 antibodies and anti-human IL-8 monoclonal antibodies (mAb; WS4) were used. The concentrations of IL-10 and TGF- β were determined by means of ELISA, purchased from BioSource International, Inc. (Camarillo, CA). Determination of the cytokine level in a culture supernatant was carried out in duplicate. The detection limits for IL-8, IL-10, and TGF- β were 20 pg/ml, 11 pg/ml, and 8 pg/ml, respectively. For TGF- β determination, samples were not extracted with acid ethanol so that a latent inactive precursor rich in serum was not detected. Moreover, the values for given amounts of an active form of human TGF- β supplied by a manufacturer in the presence of human serum were almost identical with those in the absence of human serum (e.g., 40.0 pg/ml vs. 39.7 pg/ml), indicating that only an active form of TGF- β was detected with this assay.

Cytokines and antibodies

mAb to Fc γ RI clone 22 (M22, IgG1) were provided by Dr. Michael W. Fanger (Department of Microbiology and Medicine, Dartmouth Medical School, Lebanon, NH) [10]. Anti-human IL-10 mAb (IgG2b), chicken anti-human TGF- β antibodies (IgY), normal mouse IgG2b, and normal chicken IgY were purchased from R & D Systems (Minneapolis, MN).

Statistics

The significance between groups was assessed by Student's *t*-test.

RESULTS

Potentialiation by HS of anti-inflammatory cytokine production by PMA-treated THP-1 cells in response to apoptotic cells

To examine the production by human macrophages of anti-inflammatory cytokines IL-10 and TGF- β in response to apoptotic cells, we used PMA-treated THP-1 cells and human monocyte-derived macrophage in this study. When apoptotic CTLL-2 cells were cocultured with PMA-treated THP-1 cells in the presence of FCS for 3 h, TGF- β and IL-10 production was augmented minimally in comparison with culturing PMA-treated THP-1 cells alone (Fig. 1A). In contrast, HS greatly potentiated IL-10 and TGF- β production (Fig. 1A). Such potentiation by HS was not observed when PMA-treated THP-1 cells were cultured alone (Fig. 1A) or with normal CTLL-2 cells (unpublished results). We chose the coculturing time of 3 h because phagocytosis was completed within this time period [7].

We then examined the effect of HS on the production of proinflammatory cytokine, IL-8, by PMA-treated THP-1 cells in response to apoptotic cells. When cocultured in the presence of FCS, the significant level of IL-8 protein was detected in the supernatant (Fig. 1B). In contrast, the addition of HS instead of FCS inhibited the IL-8 production significantly (Fig. 1B). The time-course study for IL-8 production by PMA-treated THP-1 cells and monocyte-derived macrophages in response to apoptotic cells revealed that IL-8 production was completed within 3 h (Fig. 1C), being consistent with the time course of phagocytosis described above. PMA-treated THP-1 cells did not produce any detectable amounts of IL-8 in the absence of apoptotic CTLL-2 cells or in the presence of normal apoptotic CTLL-2 cells (i.e., <20 pg/ml). Although the apoptotic CTLL-2 cell membranes appeared to be intact as assessed by the release of lactate dehydrogenase—a cytosolic enzyme, the possibility remains that during the coculture, the apoptotic CTLL-2 cells became secondarily necrotic to induce the IL-8 production. We therefore examined whether necrotic CTLL-2 cells induce IL-8 production by PMA-treated THP-1 cells and whether HS affects the IL-8 production by PMA-treated THP-1 cells in response to necrotic CTLL-2 cells. As shown in Figure 1B, although necrotic CTLL-2 cells induced IL-8 production, HS could not inhibit the IL-8 production by PMA-treated THP-1 cells in response to necrotic CTLL-2 cells. This was also true for necrotic P388 cells (110.5 ± 1.2 ng/ml for FCS vs. 109.5 ± 3.5 ng/ml for HS), suggesting that the suppressive effect of HS on the IL-8 production is restricted to the response of macrophages to apoptotic cells. Therefore, subsequent study was focused on how HS suppressed IL-8 production of human macrophages in response to apoptotic cells.

To determine whether the anti-inflammatory cytokines thus produced are responsible for the suppression of the IL-8 production, we included anti-IL-10 mAb (0.12 μ g/ml), anti-TGF- β antibodies (40 μ g/ml), or both in a coculture of PMA-treated THP-1 cells with apoptotic CTLL-2 cells. These antibodies were used at the concentrations enough to inhibit the highest amount of each cytokine detected in the supernatant,

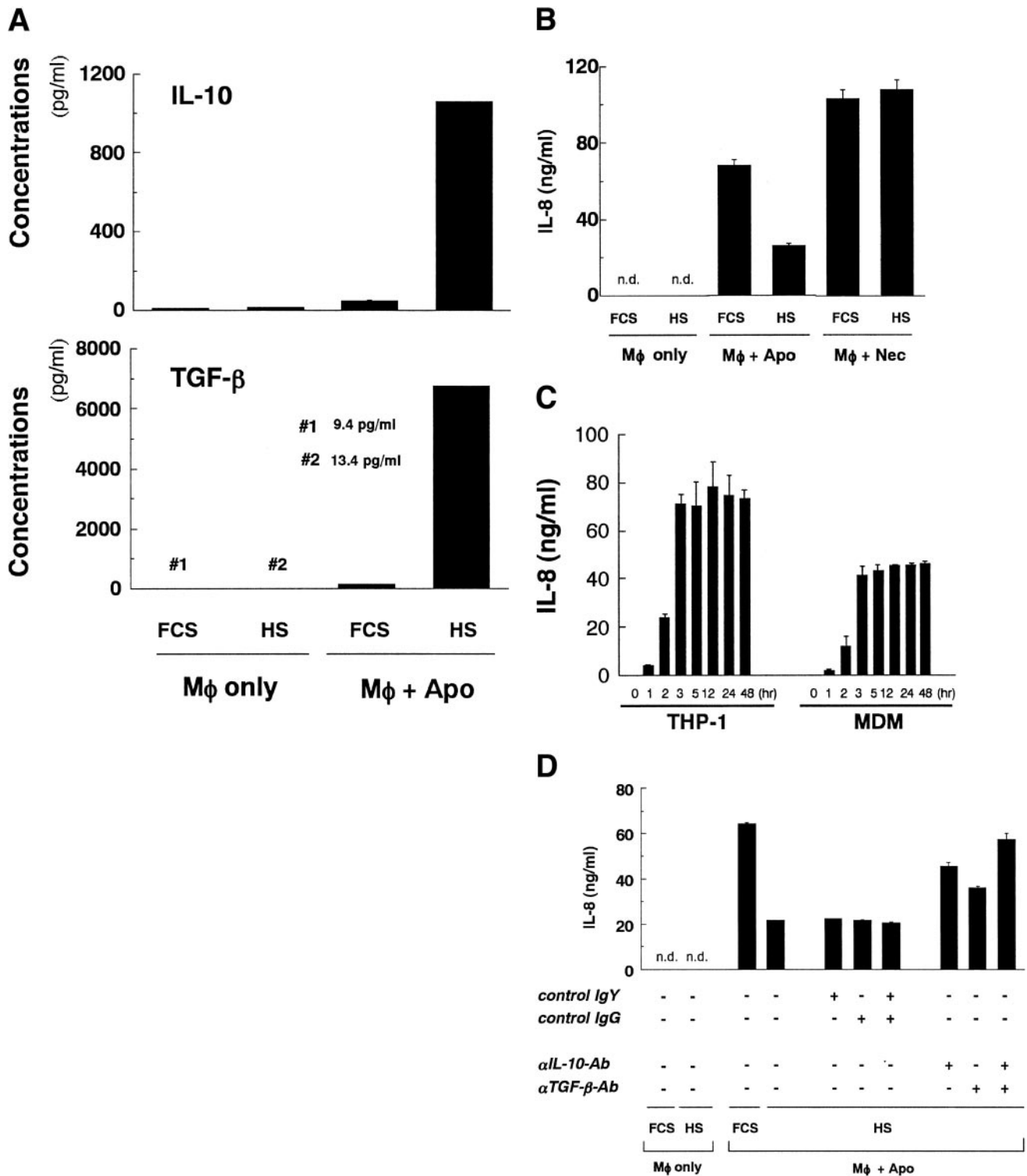


Fig. 1. (A) Potentiation by HS of anti-inflammatory cytokine production by PMA-treated THP-1 cells in response to apoptotic cells. PMA-treated THP-1 cells were cultured for 3 h with apoptotic CTLL-2 cells in the presence of 10% FCS or 10% HS. The IL-10 and TGF- β proteins in each supernatant were quantitated by means of specific ELISAs. Experiments were carried out in triplicate. The results are expressed as the means \pm SE. M ϕ , Macrophage. (B) Effects of HS on IL-8 production by PMA-treated THP-1 cells in response to apoptotic or necrotic CTLL-2 cells. Apoptotic or necrotic CTLL-2 cells were cocultured with PMA-treated THP-1 cells for 3 h in the presence of 10% FCS or 10% HS, followed by IL-8 protein measurement by means of a specific ELISA. The results are expressed as the means \pm SE for a total of four cultures in two independent experiments. n.d., Not detected. (C) Time-course study for IL-8 production. PMA-treated THP-1 cells and monocyte-derived macrophages (MDM) were cultured for various time periods with apoptotic CTLL-2 cells, followed by IL-8 protein measurement by means of a specific ELISA. Experiments were carried out in triplicate. The results are expressed as the means \pm SE. (D) Effects of anti-IL-10 mAb, anti-TGF- β mAb, or both on HS-mediated suppression of IL-8 production. PMA-treated THP-1 cells were cultured for 3 h with apoptotic CTLL-2 cells in the presence of 10% FCS or 10% HS with or without each antibody. The concentrations of anti-IL-10 mAb and control IgY were 0.12 μ g/ml, and those of anti-TGF- β and control IgG were 40 μ g/ml. The IL-8 protein in each supernatant was quantitated by means of a specific ELISA. Experiments were carried out in triplicate. The results are expressed as the means \pm SE; n.d., not detected.

i.e., 1059.6 pg/ml IL-10 and 6744 pg/ml TGF- β . As shown in Figure 1D, anti-IL-10 mAb blocked the inhibitory effects of HS more efficiently than anti-TGF- β antibodies, and the combination of these antibodies blocked their inhibitory effects almost completely. Control antibodies did not influence the effects of HS.

We then examined whether our observation described above was extended to human monocyte-derived macrophages. HS caused reduction in the IL-8 production by monocyte-derived macrophages, which was abolished significantly by anti-IL-10 mAb, anti-TGF- β antibodies, or both, but not by control antibodies (Fig. 2). Indeed, HS caused increase in the IL-10 production (49.3 ± 0.1 pg/ml vs. 332.5 ± 0.1 pg/ml) and TGF- β production (113.5 ± 10.9 pg/ml vs. 724.4 ± 0.1 pg/ml) by monocyte-derived macrophages. To extend our observation described above to other types of apoptotic cells, we prepared the following apoptotic cells: CTLL-2 cells cultured in an IL-2-free medium for 12 h, P388 cells treated with etoposide for 5 h, apoptotic human neutrophils, and apoptotic murine thymocytes. The conditions for induction of apoptosis were chosen in order to keep the cells trypan blue-negative. As shown in Table 1, HS caused various degrees of reduction in the IL-8 production of PMA-treated THP-1 cells in response to these apoptotic cells, which was significantly abolished by anti-IL-10 mAb, anti-TGF- β antibodies, or both but not by control antibodies.

Because we included HS in the coculturing medium in the above experiments, we then examined the effects of HS, which was included during differentiation. As shown in Figure 3, HS caused rather more significant reduction in the IL-8 production when HS was present during differentiation. HS could not lead to further reduction in the IL-8 production when HS was included in the coculturing me-

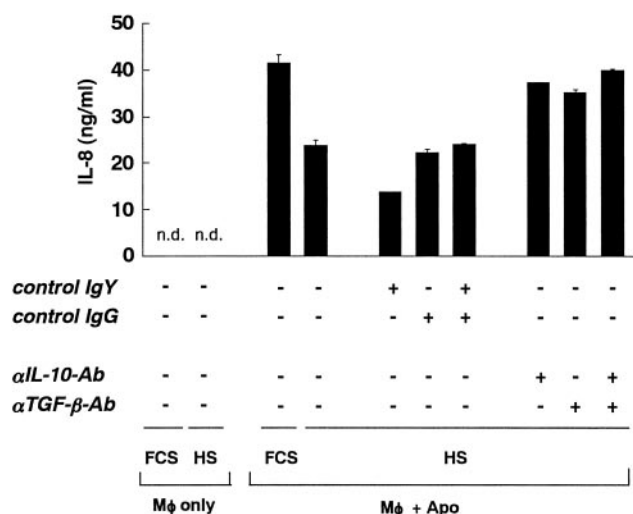


Fig. 2. Effects of anti-IL-10 mAb, anti-TGF- β mAb, or both on HS-mediated suppression of IL-8 production by monocyte-derived macrophages. Monocyte-derived macrophages were cultured for 3 h with apoptotic CTLL-2 cells in the presence of 10% FCS or 10% HS with or without each antibody. The concentration of each antibody was the same as in Figure 1C. The IL-8 protein in each supernatant was quantitated by means of a specific ELISA. Experiments were carried out in triplicate. The results are expressed as the means \pm SE; n.d., not detected.

dium and the differentiation medium. When PMA-treated THP-1 cells were pretreated with HS for 30 min, the IL-8 production in response to apoptotic cells was suppressed by 75%, whereas the suppression was not observed when apoptotic cells were pretreated with HS, suggesting that macrophages but not apoptotic cells are affected by HS.

Serum component(s) responsible for the suppression of IL-8 production

To determine which serum component(s) is responsible for HS-mediated suppression of IL-8 production, we examined the effects of hIgG and F(ab) $'_2$ of hIgG on the IL-8 production. Human IgG but not F(ab) $'_2$ of hIgG suppressed the IL-8 production (Fig. 4) and potentiated IL-10 and TGF- β production (unpublished results), suggesting that one of the components responsible for the effects on cytokine production is an Fc portion of hIgG. The extent of suppression of the IL-8 production by hIgG was somewhat smaller than that by HS. When PMA-treated THP-1 cells were pretreated with hIgG, the IL-8 production in response to apoptotic cells was suppressed by 66%, whereas the suppression was not observed when apoptotic cells were pretreated with hIgG, ruling out the possibility that hIgG may bind to apoptotic cells in order to form immune complex.

Involvement of Fc γ RI in hIgG-mediated potentiation of IL-10 and TGF- β and suppression of IL-8 production

Because Fc γ RI is the only receptor that binds to free IgG with high affinity, we then examined whether Fc γ RI is responsible for the effects of HS and hIgG.

Fc γ RI is down-modulated specifically by an anti-Fc γ RI mAb, M22 [8]. As shown in Figure 5, a and b, PMA-treated THP-1 cells expressed Fc γ RI (bold lines) and Fc γ RII (thin lines) after 48 h (Fig. 5a) and 72 h (Fig. 5b). When PMA-treated THP-1 cells were treated with M22 from 48 h to 72 h, the expression of Fc γ RI was specifically down-modulated, whereas that of Fc γ RII was unchanged (compare Fig. 5, c and b). In another set of experiments, we confirmed that the binding of clone 10.1 to Fc γ RI was not inhibited by M22 (unpublished results), excluding the possibility that M22 inhibited the binding of clone 10.1 sterically.

We then determined the potentiating and the inhibitory effects of HS and hIgG on the cytokine production after down-modulation of Fc γ RI. The effects of HS and hIgG were abolished completely on the down-modulation of Fc γ RI (Fig. 6). These results indicate that the potentiating and the inhibitory effects on the cytokine production are mediated through Fc γ RI.

DISCUSSION

It is generally believed that apoptotic cells are phagocytosed by neighboring phagocytes, such as macrophages, without inflammation [1] through the production of anti-inflammatory cytokines [3, 4]. This study demonstrated that HS potentiates the IL-10 and TGF- β production by PMA-treated THP-1 cells and

TABLE 1. Effect of Anti-IL-10 mAb and/or Anti-TGF- β Antibodies on HS-Mediated Suppression of IL-8 Production

| | FCS | | HS | | | | | | |
|-------------------------------|----------------------------|----------------|----------------|----------------------|---------------------------------------|----------------|----------------|----------------|--|
| | | | Anti-IL-10 Ab | Anti-TGF- β Ab | Anti-IL-10 Ab Anti-TGF- β Ab | IgY | IgG | IgY IgG | |
| a. M ϕ + Apo CTLL-2 12 h | 4.4 \pm 1.4 ^a | 1.3 \pm 0.5 | 4.1 \pm 0.8 | 2.8 \pm 0.2 | 4.5 \pm 1.6 | 1.4 \pm 0.2 | 1.3 \pm 0.13 | 1.9 \pm 0.4 | |
| b. M ϕ + Apo P388 cells | 12.7 \pm 0.5 | 8.1 \pm 0.7 | 9.1 \pm 1.3 | 10.2 \pm 0.2 | 11.8 \pm 0.6 | 8.2 \pm 3.4 | 8.0 \pm 0.7 | 9.1 \pm 0.4 | |
| c. M ϕ + Apo neutrophils | 74.2 \pm 4.5 | 0.8 \pm 0.1 | 74.2 \pm 0.3 | 53.8 \pm 1.5 | 75.8 \pm 2.5 | 1.2 \pm 0.1 | 1.2 \pm 0.1 | 1.0 \pm 0.7 | |
| d. M ϕ + Apo thymocyte | 57.1 \pm 4.4 | 44.4 \pm 2.8 | 49.7 \pm 0.7 | 46.7 \pm 0.3 | 56.3 \pm 1.6 | 42.8 \pm 0.6 | 43.2 \pm 0.8 | 42.6 \pm 0.1 | |

PMA-treated THP-1 cells were cultured for 3 h with apoptotic cells in the presence of 10% FCS or 10% HS, with or without each antibody. The concentrations of anti-IL-10 mAb and control IgY were 0.12 μ g/mL, and those of anti-TGF- β and control IgG were 40 μ g/mL. The IL-8 protein in each supernatant was quantitated by means of a specific ELISA. Experiments were carried out in triplicate. The results are expressed as the means \pm SE.

^a ng/mL.

monocyte-derived macrophages in response to apoptotic cells, which results in great suppression of the IL-8 production. Specific down-modulation of Fc γ RI by anti-Fc γ RI mAb, M22, led to the complete abolition of the potentiating and suppressive effects of HS and hIgG on the cytokine production, although the suppressive effect of hIgG was somewhat smaller than that of HS for unknown reasons. This strongly suggests that Fc γ RI is required for the effects of HS and hIgG and that a major component responsible for the effect of HS is IgG. Neither HS nor hIgG affected the phagocytic ability of PMA-treated THP-1 cells for apoptotic cells as assessed by flow cytometry (unpublished results). Furthermore, pretreatment of macrophages but not apoptotic cells with HS or hIgG resulted in suppression of IL-8 production, suggesting that immune complex may not be involved in the response. Thus, this study reveals a hitherto unrecognized role of hIgG and Fc γ RI in determining the balance between the level of proinflammatory cytokine and that of anti-inflammatory cytokine in macrophage response to apoptotic cells.

As most apoptotic cells used in this study are murine except human neutrophils, IL-8 is clearly produced by macrophages but not apoptotic cells. Moreover, we did not detect any macrophage inflammatory protein-2 and IL-8 in the supernatants of apoptotic cells including apoptotic CTLL-2 cells and apoptotic human neutrophils, which contrasted with a previous study [11].

HS led to various degrees of suppression of IL-8 production by not only PMA-treated THP-1 cells but also by human monocyte-derived macrophages (unpublished results) in response to apoptotic cells. Because the IL-8 production in response to necrotic cells was not suppressed by HS (Fig. 1B), it is possible that these apoptotic cells may contain (secondary) necrotic cells to various extents. Such a possibility is rather remote, however, because all of apoptotic cells used in Table 1 were trypan blue-negative and because the coculturing time (3 h) was not long enough to induce secondary necrosis. Multiple ligands and receptors are involved in the interaction between macrophages and apoptotic cells [12], and therefore it is possible that IL-8 production and its suppression by HS may depend on a combination of ligand and receptor.

Many studies have demonstrated that IL-10 is a potent anti-inflammatory cytokine. In one study, IL-10 inhibited IL-8

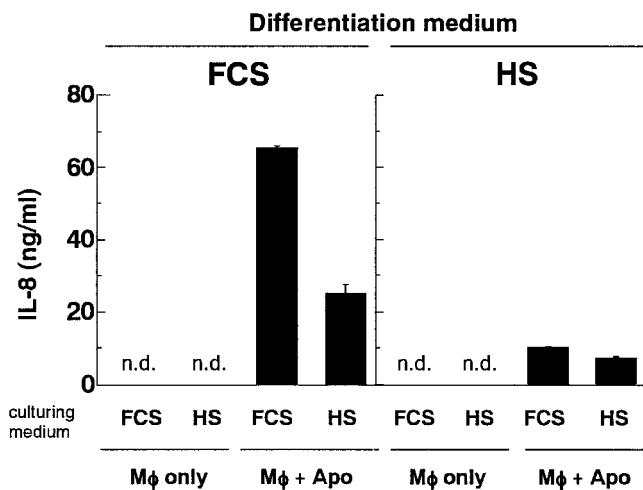


Fig. 3. Effects of addition of HS in the differentiation medium on IL-8 production. PMA treatment of THP-1 cells was carried out in the presence of FCS or HS, followed by washing with PBS twice. Then, apoptotic CTLL-2 cells were cocultured with PMA-treated THP-1 cells for 3 h in the presence of 10% FCS or 10% HS, followed by IL-8 protein measurement by means of a specific ELISA. The results are expressed as the means \pm SE for a total of four cultures in two independent experiments. n.d., not detected.

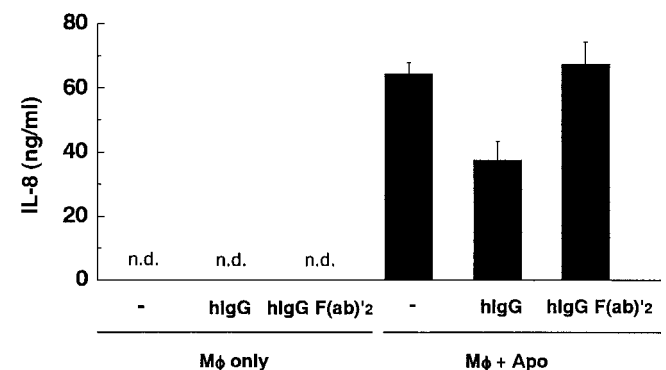


Fig. 4. Effects of hIgG or F(ab)₂ of hIgG on IL-8 production by PMA-treated THP-1 cells in response to apoptotic CTLL-2 cells. Apoptotic CTLL-2 cells were cocultured with PMA-treated THP-1 cells for 3 h in the absence or presence of 5 mg/ml hIgG or 5 mg/ml F(ab)₂ of hIgG, followed by IL-8 protein measurement by means of a specific ELISA. The results are expressed as the means \pm SE for a total of four cultures in two independent experiments. n.d., Not detected.



Fig. 5. Down-modulation of FcγRI. THP-1 cells were cultured with PMA for 48 h (a) or 72 h (b). (c) THP-1 cells were cultured with PMA for 72 h and treated with 0.1 μg/ml M22 from 48 to 72 h. After the cultures, the cells were analyzed for the expression of FcγRI (thick-lined, open histograms) or FcγRII (thin-lined, open histograms). The control profiles without staining are shown as filled histograms.

production at a later period in polymorphonuclear leukocytes triggered by LPS [13]. In another study, IL-10 was produced through ligation of macrophage FcγRI with opsonized pathogens and inhibited IL-12 production [14], this being another example that FcγRI plays a role in IL-10 production. In yet another study, polymorphonuclear leukocytes were accumulated within the mouse peritoneal cavity in response to the administration of zymosan, and this response was inhibited by endogenously produced IL-10 [15]. Therefore, our study extends these observations to the extent that IL-10 is also produced through ligation of macrophage FcγRI with normal IgG when the macrophages phagocytose apoptotic cells. If apoptotic cells were not provided for the macrophages, the production of neither IL-10 nor TGF-β was induced by HS and hIgG. Thus, the production of IL-10 and TGF-β requires the ligation of FcγRI and the binding of apoptotic cells under our conditions.

We do not have any direct evidence indicating that the mechanism described in this study operates *in vivo*. However, there are several reports in which the mechanism described in this study may be involved. In one study dealing with a clinically relevant model of sepsis, cecal ligation and puncture, overexpression of Bcl-2 prevented lymphocyte apoptosis and improved survival from sepsis, and Rag-1^{-/-} mice showed decreased survival compared with immunologically normal mice with sepsis [16]. This might indicate the importance of down-modulation of inflammatory reactions in sepsis by IgGs, although the authors suggested the important role of lymphocytes in the host defense in sepsis [16]. In another study, TGF-β^{-/-} mice did not show any abnormality in development, but approximately 20 days after birth, they developed a multifocal inflammatory disease not as a result of primary infection by common mouse pathogens [17]. This might be due to dysregulation of the inflammatory response after phagocytosis of apoptotic cells, although the authors suggested an autoimmune response as a possible cause. In another study, IL-10^{-/-} mice developed chronic enterocolitis, which the authors suggested was a result of uncontrolled immune responses triggered by enteric antigens [18]. Although our explanations for these findings are entirely speculative, we believe that the mechanism described in this study sheds light on the pathology and physiology in association with extensive apoptotic death. For instance, the mechanism may operate in the liver and the spleen, where numerous apoptotic blood cells, such as aged neutrophils, are taken up by phagocytes without inflammation. Notably, the IL-8 production of human macrophage in response

to apoptotic neutrophils was suppressed most greatly by HS, which may prevent apoptotic neutrophils from inducing infiltration of neutrophils.

We have reported the IL-8 production by human macrophages, PMA-treated THP-1 cells, as well as monocyte-derived macrophages in response to apoptotic CTLL-2 cells [5], which was quite contrasted with the generally held view. Several possibilities have been raised for our previous findings. For instance, our apoptotic CTLL-2 cells may have contained secondary necrotic cells. Such IL-8 production may be limited to apoptotic CTLL-2 cells. However, these possibilities are very unlikely, because IL-8 production was not restricted to late apoptotic CTLL-2 cells as shown in this study. Now this study may explain the discrepancy between our previous result [7] and other results [2, 4], although apoptotic cells still induced IL-8 production in the presence of HS. We obtained human monocyte-derived macrophages by culturing in the presence of

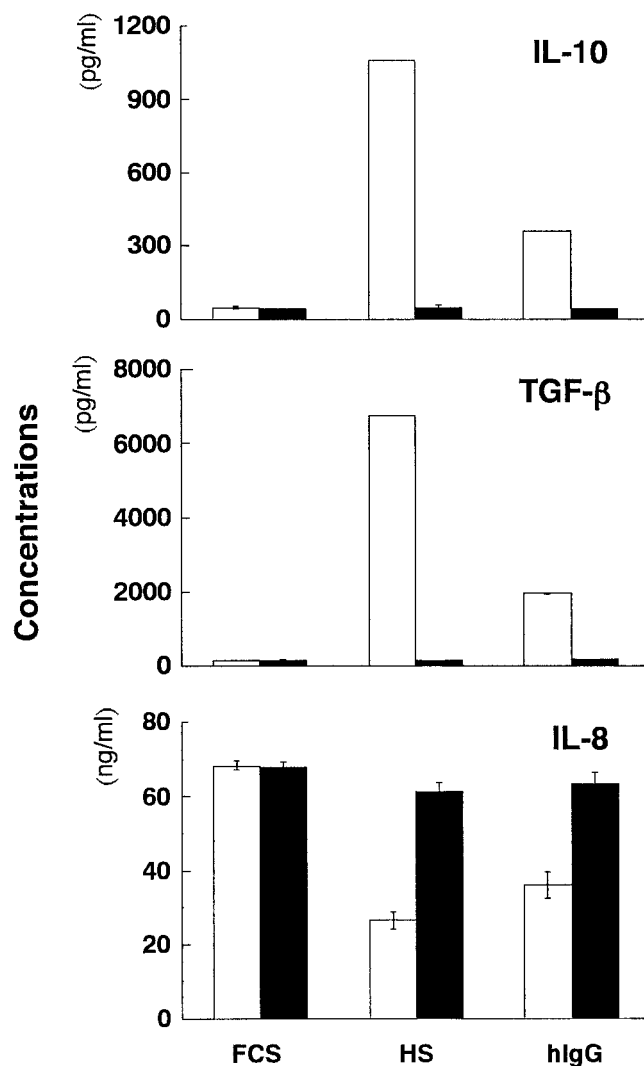


Fig. 6. Effects of down-modulation of FcγRI on cytokine production. PMA-treated THP-1 cells with (solid bars) or without (open bars) down-modulation of FcγRI were cultured for 3 h with apoptotic CTLL-2 cells in the presence of FCS, HS, or FCS containing hIgG, followed by IL-10, TGF-β, and IL-8 protein measurements by means of specific ELISAs. The results are expressed as the means ± SE for a total of four cultures in two independent experiments.

M-CSF and FCS, whereas other researchers obtained them by culturing in the presence of HS [2, 4]. The reason why we have not used HS for differentiation is that in our hands, M-CSF and FCS produced monocyte-derived macrophages more reproducibly than HS. Although most macrophages might be differentiated from monocytes under the condition rich in serum, some macrophages might move to tissues not surrounded with serum, such as thymus and skin, where down-regulation of IL-8 production by HS may have pathological and physiological relevance.

In this study, we demonstrated a mechanism by which IL-8 production in response to apoptotic cells can be prevented. Because this mechanism did not always lead to complete shutting off of the proinflammatory cytokine production, there must be an as-yet unknown mechanism(s) that complements the mechanism described in this study. One such mechanism(s) may be that macrophages produce little or no inflammatory cytokine in response to the much earlier stage of apoptotic cells, as suggested by our previous study [7] as well as this study. In conclusion, this study suggests the possibility that normal hIgG plays a critical role in the noninflammatory clearance of apoptotic cells in humans.

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