Interaction of Lactoferrin, Monocytes, and T Lymphocyte Subsets in the Regulation of Steady-State Granulopoiesis In Vitro

GROVER C. BAGBY, JR., VASILIKI, D. RIGAS, ROBERT M. BENNETT, ARTHUR A. VANDENBARK, and HARINDER S. GAREWAL, with the technical assistance of BRENDA WILKINSON and JULIE DAVIS, *The Medical and Surgical Research Laboratories, Veterans Administration Medical Center; The E. E. Osgood Memorial Center Laboratory, and Divisions of Hematology/Oncology and Immunology/Allergy/Rheumatology, University of Oregon Health Sciences Center, Portland, Oregon 97201*

ABSTRACT Colony-stimulating activities (CSA) are potent granulopoietic stimulators in vitro. Using clonogenic assay techniques, we analyzed the degree to which mononuclear phagocytes and T lymphocytes cooperate in the positive (production/release of CSA) and feedback (inhibition of CSA production/release) regulation of granulopoiesis. We measured the effect of lactoferrin (a putative feedback regulator of CSA production) on CSA provision in three separate assay systems wherein granulocyte colony growth of marrow cells from 22 normal volunteers was stimulated by (*a*) endogenous CSA-producing cells in the marrow cell suspension, (*b*) autologous peripheral blood leukocytes in feeder layers, and (*c*) medium conditioned by peripheral blood leukocytes. The CSA-producing cell populations in each assay were varied by using cell separation techniques and exposure of isolated T lymphocytes to methylprednisolone or to monoclonal antibodies to surface antigens and complement. We noted that net CSA production increased more than twofold when a small number of unstimulated T lymphocytes were added to monocyte cultures. Lactoferrin's inhibitory effect was also T lymphocyte dependent. The T lymphocytes that interact with monocytes and lactoferrin to inhibit CSA production are similar to those that augment CSA production because their activities are neither genetically restricted nor glucocorticoid sensitive, and both populations express HLA-DR (Ia-like) and T3 antigens but not T4 or T8 antigens. These findings are consistent with results of our studies on the mechanism of lactoferrin's inhibitory effect which indicate that mononuclear phagocytes produce both CSA and soluble factors that stimulate T lymphocytes to produce CSA, and that lactoferrin does not suppress monocyte CSA production, but does completely suppress production or release by monocytes of those factors that stimulate T lymphocytes to produce CSA. We conclude that mononuclear phagocytes and a subset of T lymphocytes exhibit important complex interactions in the regulation of granulopoiesis.

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INTRODUCTION

Clonal growth in vitro of committed granulopoietic progenitor cells is dependent upon the presence in the culture system of a family of glycoprotein molecules known collectively as colony-stimulating activity (CSA)[1] (1). Although a variety of cell types may produce CSA (1), the most widely studied of these are mononuclear phagocytes (2, 3) and mitogen-stimulated thymus-derived lymphocytes (T lymphocytes) (4, 5). Although both of these cell types are thought to be capable of independently producing CSA, recent evidence suggests that when T lymphocytes and monocytes are cultured together under conditions of in vitro stimulation, they interact to produce more CSA than would be predicted by summing the CSA produced in cultures of the isolated cell types (6, 7). Our observations further demonstrate that T lymphocytes and monocytes

[1] *Abbreviations used in this paper*: CSA, colony-stimulating activities; FCS, fetal calf serum; LDBMC, low density bone marrow cells; PBLM, peripheral blood mononuclear leukocytes; T lymphocytes, thymus-dependent lymphocytes.

interact in this way even in the absence of in vitro stimulation.

Feedback or negative, regulatory influences on granulocyte colony growth are less well understood. Recently it was suggested that lactoferrin, an iron-binding protein found in the specific granules of neutrophils (8), may function as a feedback regulator of granulopoiesis (9, 10). Broxmeyer (11) has proposed that lactoferrin binds to those mononuclear phagocytes that possess Ia-like antigens, and directly inhibits the production of CSA by those cells. We have confirmed many of Broxmeyer's observations: that lactoferrin is the activity in neutrophil extracts that effects inhibition of production or release of CSA by mononuclear leukocytes, and that lactoferrin concentrations as low as 0.01 fM are active (12). However, we report that using techniques that afford monocyte and T lymphocyte separation, lactoferrin does not inhibit net CSA production by monocytes from either the peripheral blood or bone marrow. Rather, lactoferrin inhibits the production by monocytes of a soluble factor that simulates T lymphocytes to produce CSA. The T lymphocytes that interact with monocytes and lactoferrin to inhibit CSA production are cortisol resistant and not genetically restricted, and express HLA-DR (13, 14) and T3 (15, 16) antigens but not T4 or T8 antigens.

METHODS

Preparation of marrow and peripheral blood cell populations. Bone marrow and peripheral blood cells were obtained in heparinized syringes from 21 informed and consenting normal adult volunteers. Low density bone marrow cells (LDBMC) and peripheral blood mononuclear leukocytes (PMBL) were prepared by centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Division of Pharmacia, Inc., Piscataway, N. J.) as described (17). Cell suspensions were depleted of monocytes using a carbonyl iron/magnet technique. Suspensions were depleted of T lymphocytes by placing cell suspensions incubated with washed sheep erythrocytes (Prepared Media Laboratories, Tualatin, Ore.) on Ficoll-Paque (17). When the nonrosetting cells were again exposed to sheep erythrocytes 0–2% formed E rosettes. The rosetting cells contained 0–3% monocytes by Wright-Giemsa and alpha naphthyl butyrate esterase stains. T lymphocyte suspensions were prepared from the rosetting population by gentle agitation and osmotic lysis (18). Monocytes were prepared by incubating PBML or LDBMC in 60-mm plastic tissue culture dishes that had been coated with lactoferrin-depleted and heat-inactivated fetal calf serum (FCS) overnight at 4°C with subsequent removal of the nonadherent cells (19). The monocytes were either covered with agar medium or lifted off the dish using 2% lidocaine medium followed by three washes with complete medium (19). In some instances, 0.2% EDTA in saline with 5% FCS was used instead of lidocaine. These monocyte suspensions were 92% monocytes by Wright's and alpha naphthyl butyrate esterase stains and >98% viable by trypan blue dye exclusion. They phagocytosed latex particles, oxidized ^{14}C-glucose in response to particulate challenge, expressed Fc receptors, and produced CSA in vitro (see below). Monocyte suspensions contained <2% E-rosetting cells. In feeder layer studies and with limiting dilution studies, the monocytes were further subjected to sheep erythrocyte rosette depletion before use. Those cell suspensions contained no detectable E-rosetting cells. Monocyte-depleted PBML contained 0–4% esterase-positive cells and from 25 to 68% T lymphocytes (E-rosetting cells).

Lactoferrin and lactoferrin antibodies. Highly purified human breast milk lactoferrin and rabbit antihuman lactoferrin antibodies were prepared as described (20). The lactoferrin preparation is free of endotoxin (12) and our previous studies suggest that the preparation is free of contaminants (20). We used both native lactoferrin (8–10% iron saturated) and highly saturated (70–75%) lactoferrin in the assays below. Because the removal of iron from lactoferrin in low pH solutions results in artifactual changes in the structure of the protein (21), we did not use iron-depleted (apo) lactoferrin in this study. Antilactoferrin antisera were kept at −70°C before use.

Colony-inhibiting activity assays

Leukocyte feeder layers. PBML or subpopulations thereof obtained from normal volunteers were used to prepare feeder layers in 0.5% agar medium (McCoy's 5a with 15% lactoferrin-depleted FCS, Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.). The plates contained lactoferrin (0.01, 1 pM), or no lactoferrin. Upon each feeder layer was placed 0.3–2 × 10^5 phagocyte-depleted, T lymphocyte-depleted LDBMC in 1 ml of 0.9% methylcellulose in alpha medium with 15% lactoferrin-depleted FCS. Colonies (aggregates >40 cells) and clusters (aggregates of >8 and <40 cells) were counted after 7 d of culture in a fully humidified atmosphere of 7.5% CO_2 in air at 37°C.

Leukocyte-conditioned medium. PBML or fractions thereof were cultured in 1-ml suspensions in RPMI 1640 (Gibco Laboratories) with 15% lactoferrin-depleted FCS for 7 d in the presence and absence of 0.01 pM lactoferrin. The conditioned media were harvested and added (10% vol/vol) to single-layer methylcellulose or agar cultures as described (17). Target cells were again T-depleted, phagocyte-depleted LDBMC from the volunteer whose leukocytes were used to prepare the conditioned medium.

Spontaneous colony growth. LDBMC or fractions thereof were cultured in methylcellulose (0.9%). No exogenous source of CSA was added. Thus, "spontaneous" colony growth is that which is stimulated by CSA-producing cells in the plated marrow sample. In addition, using cells from five normal volunteers, phagocyte-depleted, T cell-depleted LDBMC were plated with and without lactoferrin and before and after adding 0.5–1.0 × 10^5 T lymphocytes, marrow monocytes, peripheral blood monocytes, or T lymphocytes (0.5 × 10^5/ml) plus monocytes (0.5 × 10^5/ml). Furthermore, mixing experiments were done wherein phagocyte-depleted LDBMC from one volunteer were mixed with T lymphocytes and monocytes obtained from a genetically unrelated volunteer. This mixing experiment was done four times.

Treatment of T lymphocytes with monoclonal antibodies. 0.1 ml T lymphocytes (10 × 10^6/ml) was mixed with 0.1 ml monoclonal antibodies and incubated 20 min at 22°C. 0.5 ml rabbit complement was added, and the suspension was cultured at 37° × 60 min. The cells were washed three times in complete medium and were used in combination with other leukocytes in one of the three clonogenic assays described above. The monoclonal antibodies used were 1:25 dilutions of OKT3, OKT4, and OKT8 (Ortho Pharmaceutical Corp., Raritan, N.J.) and 1:16, 1:64, and 1:128 dilutions of an antibody against monomorphic DR (Ia-like) determinants known as E18/3(22). This experiment was performed six times using cells from six normal volunteers. Cytofluorographic analysis

of both PBML and T lymphocytes was performed by indirect immunofluorescence with fluorescein-conjugated goat anti-mouse IgG (N. L. Cappel Laboratories Inc., Cochranville, Pa.). Specifically, 10^6 cells were treated with one of the four monoclonal antibodies at 4°C for 30 min. Antibody dilutions tested were 1:5, 1:8, and 1:16. The cells were washed twice, treated with 1:40 dilution of the goat anti-mouse IgG for 30 min at 4°C, and washed again. The cells were then analyzed on the Cytofluorograf FC-210 (Ortho Instruments, Westwood, Mass.). The intensity of fluorescence was recorded on a pulse-height analyzer. Background staining was analyzed on cells treated with the second antibody alone.

Treatment of T lymphocytes with methylprednisolone. 10×10^6 T lymphocytes/ml were suspended in complete medium for 80 min at 37°C (7.5% CO_2 in air) with 0.1, 1, or 10 μM methylprednisolone sodium succinate (Upjohn Co., Kalamazoo, Mich.) (23). The cells were washed twice, resuspended in original volume, and used alone or in combinations with other cell populations in each of the three clonogenic assays described above.

Experimental Design

Effect of lactoferrin on spontaneous colony growth. 1×10^5 monocytes from peripheral blood and bone marrow were separately added to T-depleted, phagocyte-depleted LDBMC from the same volunteer in single layer assays (methylcellulose) that contained 10 nM, 0.1 nM, 1 pM, 0.01 pM, or no lactoferrin. Other plates were simultaneously made with the same T-depleted, phagocyte-depleted targets plus both monocytes (10^5) and T cell (10^5) in the same plate. In some cases T cells were treated with monoclonal antibody and complement (see above) or with methylprednisolone (see above). Colonies and clusters were counted on day 7 of culture. Cells from 10 normal volunteers were studied. Co-culture studies were performed as described using cells from four volunteers.

Effect of lactoferrin on CSA and other factors in leukocyte-conditioned medium. 1×10^5 peripheral blood monocytes/ml or 10^5 T lymphocytes/ml were cultured in RPMI-1640 with 15% FCS for 5–7 d with and without lactoferrin. Other 1-ml plates contained 1×10^5 monocyte-depleted PBML, 1×10^5 T cells, or mixtures (as above) of monocytes and T cells. Conditioned media were harvested and used (5 and 10% vol/vol) on the day of harvest (Millipore filtration was not performed, nor were the conditioned media frozen before the first assay) to stimulate colony growth of autologous T-depleted, phagocyte-depleted LDBMC. Colonies and clusters were counted on day 7 of culture (as above). Cells from nine normal volunteers were studied.

Leukocyte-conditioned media were also tested for factors that might stimulate CSA production by other cells. Specifically, 10^5 autologous T lymphocytes/ml were suspended in medium containing 10, 30, or 50% conditioned medium prepared using monocytes or monocytes plus lactoferrin 0.01 pM. The T lymphocyte-conditioned media were assayed for CSA after 5 d in culture as above. Control media were 10, 30, and 50% monocyte-conditioned media kept in 1-ml plates at 37°C in 7.5% CO_2 in air for 5 d. Conversely, autologous monocytes (10^5/ml) were suspended in culture medium containing 10, 30, or 50% T lymphocyte-conditioned medium. The monocyte-conditioned medium was harvested after 5 d of culture under the conditions described and was analyzed for CSA content as above. These studies were performed to test the hypothesis that one population of leukocytes (e.g., monocytes) produces factors that stimulate other populations (e.g., lymphocytes) to produce CSA and that lactoferrin inhibits the production of these factors. In these, as in other CSA assays, a portion of the target cell sample was stimulated with human placental-conditioned medium to ensure that the test CSA was at subplateau levels.

Effect of lactoferrin on CSA production release by cells in feeder layers. 5×10^5 peripheral blood monocytes were placed in 1 ml 0.5% agar feeder layers (as above) with and without 0.01 pM lactoferrin and were used to stimulate phagocyte-depleted, T-depleted LDBMC. Colonies and clusters were counted after 7 d of bone marrow cell incubation.

Limiting dilution studies were performed by modifying the feeder assays so that T lymphocytes of increasing numbers were added to a fixed number of autologous peripheral blood monocytes in 0.5% agar-medium feeder layers. Each 1-ml feeder layer contained 5×10^5 monocytes, and a variable number of T lymphocytes (0, 10^3, 5×10^3, 10^4, 5×10^4, 10^5, 5×10^5), and either 0.01 pM lactoferrin or no lactoferrin. After 24 h of incubation, 0.3×10^5 T-depleted, phagocyte-depleted LDBMC in methylcellulose and alpha medium (as above) were layered upon the feeder layers. Colonies and clusters were counted after 7 d of marrow cell culture. All studies were done in four or five replicate plates. Enhancement or inhibition of colony growth in the observed plates was greater or less than control colony growth with $P < 0.05$ (Student's t test).

RESULTS

Initially, multiple doses of lactoferrin were tested in these assays, and we found that 0.01 pM–1 aM were universally effective in inhibiting CSA production or release (12). We carried out the remainder of the studies using 0.01 pM lactoferrin. Lactoferrin failed to inhibit CSA production/release by monocytes in suspension culture (Fig. 1). Monocyte-depleted PBML provided little CSA in conditioned medium, and lactoferrin had no inhibitory effect. However, when monocytes and monocyte-depleted PBML were recombined, CSA in conditioned medium increased to a greater extent than would be expected by the summation of CSA from the two sources cultured alone. Moreover, lactoferrin inhibited CSA provision in conditioned medium when monocytes and monocyte-depleted PBML were combined.

We tested the hypothesis that the critical nonmonocyte population was a T lymphocyte or a subset thereof. We noted that in the spontaneous colony growth assay (no exogenous CSA added), lactoferrin inhibited colony growth only when both mononuclear phagocytes and T lymphocytes were present together as the source of CSA (Fig. 2A). In addition, consistent with the observations in conditioned medium assays, CSA provision was maximal when both monocytes and T cells were present. In Fig. 2B similar results are noted. For example, maximal clonal growth was noted only when monocytes and T cells were present together and only then did lactoferrin inhibit clonal growth. When monocytes were added back to LDBMC depleted of both phagocytes and T lymphocytes, colony growth increased but lactoferrin failed to reduce colony growth. However, when both T lympho-

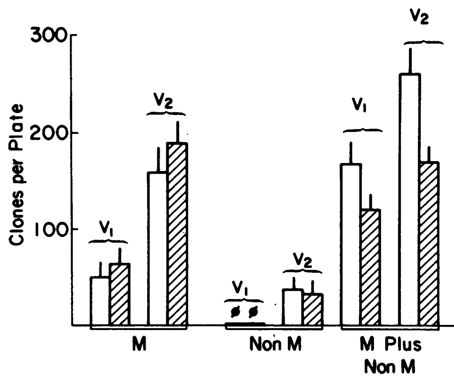
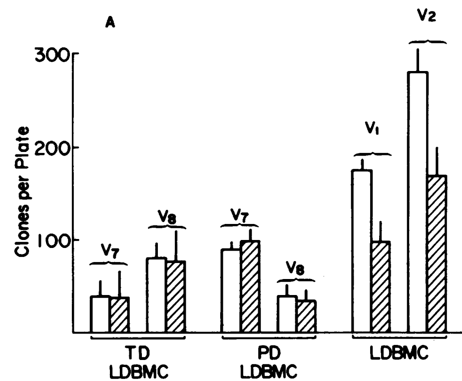


FIGURE 1 The effect of 0.01 pM lactoferrin (cross-hatched bars) on CSA production by leukocytes in suspension culture. CSA-producing cells were obtained from two volunteers, V_1 and V_2 and 10% leukocyte-conditioned medium was tested in 1 ml methylcellulose culture with 1×10^5 autologous T lymphocyte-depleted, phagocyte-depleted LDBMC. The results shown are from two to six studies. Results are expressed as mean±SD clones (colonies plus clusters) per plate. M represents colony growth stimulated by enriched monocytes (92% esterase positive, 1% E rosette positive). Non-M represents monocyte-depleted PBML. M plus non-M (V_1 $P < 0.01$; V_2 $P < 0.01$) represents a remixture of monocytes and nonmonocytes. In both cases the addition of non-M to M-enhanced CSA production. Moreover, lactoferrin failed to inhibit CSA production or release by M and non-M but did effect inhibition when M and non-M were present together. Cells from four other normal volunteers were similarly studied, and the results were the same.

cytes and monocytes were added back, even more clonal growth was seen, and lactoferrin significantly inhibited it.

We tested the ability of allogeneic T cells to participate in the phenomenon of lactoferrin responsiveness by mixing phagocyte and T lymphocyte-depleted LDBMC with both autologous and allogeneic monocytes or T lymphocytes in a spontaneous clonal growth assay. The results (Fig. 3) indicate that T cells and monocytes cooperate in CSA provision and in responsiveness to lactoferrin, and the cooperative interaction is not restricted to genetically identical cells. Two additional studies showed similar results (data not shown). Fig. 4 displays the combined results of 20 studies using two assay techniques. Although slight lactoferrin-mediated inhibition of CSA production/ release by monocytes was noted occasionally, consistent and high level inhibition has only been seen when T cells and monocytes were cultured together.

We tested some of the surface antigenic characteristics of the cooperating T cells by performing monocyte/T cell mixing experiments. In cytotoxicity assays OKT3 killed 85–90% T lymphocytes in all studies. OKT4 and OKT8 killed 50–62 and 35–41% T lymphocytes, respectively. E18/3 killed 0–5% T cells. On cytofluorographic analysis 75 and 9% T lymphocytes exhibited fluorescence after exposure to OKT3 (1:8)



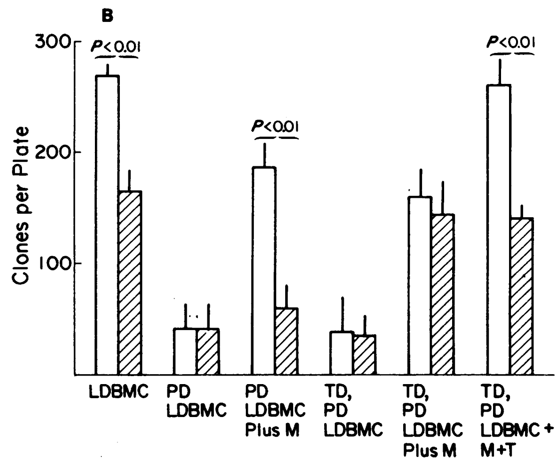


FIGURE 2 Effect of lactoferrin (0.01 pM cross-hatched bars) on spontaneous clonal growth of LDBMC before and after depletion of either T lymphocytes (TDLDBMC) or phagocytes (PDLDBMC) from the plated cell suspension. Two (V_7 and V_8) of four studies are shown here. Bars and vertical lines represent mean±SD in four to five replicate plates. LDBMC were 18% (V_7) and 22% (V_8) esterase positive. PDLDBMC were <2% esterase positive. (A) Commensurate with observations noted in Fig. 1, clonal growth was highest when T lymphocytes and phagocytes were present together. Moreover, lactoferrin inhibited colony growth only when both T lymphocytes and phagocytes were present in suspension and not when clonal growth was stimulated by either T cells or monocytes alone. Spontaneous clonal growth of T-depleted and phagocyte-depleted LDBMC was nil. V_1, $P < 0.01$; V_2, $P < 0.01$. (B) One (V_{13}) of two studies (spontaneous clonal growth assay) where either M (0.4×10^5 monocytes/plate) or T (0.4×10^5 T lymphocytes/plate) plus monocytes (0.4×10^5/plate) were added to 0.4×10^5 phagocyte-depleted LDBMC (TD, PDLBMC) before plating with (cross-hatched bars) and without (open bars) 0.01 pM lactoferrin. In this study lactoferrin inhibits clonal growth of LDBMC only in the presence of both M and T.

and E18/3 (1:5), respectively. The results shown in Fig. 5 indicate that when monocytes and control T cells were mixed, colony growth was enhanced. Lactoferrin inhibited colony control. When T3 and E18/3 positive cells were removed, clonal growth was not enhanced over that stimulated by monocytes alone, and lacto-

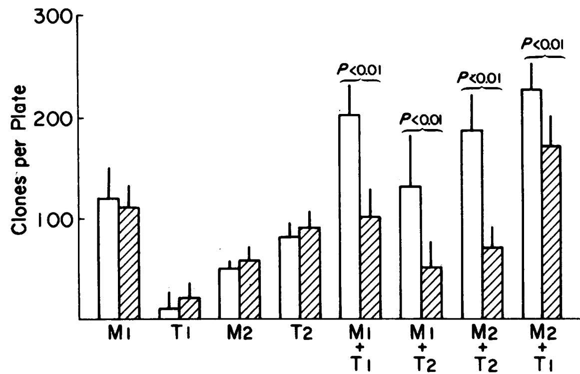


FIGURE 3 The inhibitory effects of lactoferrin on CSA production by mixtures of monocytes and T lymphocyte from two normal volunteers: M_1 represents monocytes from one volunteer and M_2 represents monocytes from another volunteer, not genetically related. The effect of lactoferrin 0.01 pM (cross-hatched bars) on clonal growth of 1×10^5 T lymphocyte and phagocyte-depleted LDBMC (from V_{18}) per plate to which had been added monocytes ($M_1 = 0.5 \times 10^5$/plate, $M_2 = 1.1 \times 10^5$/plate) or T lymphocytes ($T_1 = 0.5 \times 10^5$/plate, $T_2 = 0.6 \times 10^5$/plate) or both is shown above where bars and vertical lines represent mean±SD, respectively. This is one example of three studies, each of which uses cells from two genetically unrelated donors. The mixture of monocytes and T cells effected an increase in CSA production (compared with M or T alone or M + T). Lactoferrin inhibited CSA production by all mixtures of M + T but not M or T alone. The findings in two additional studies were the same.

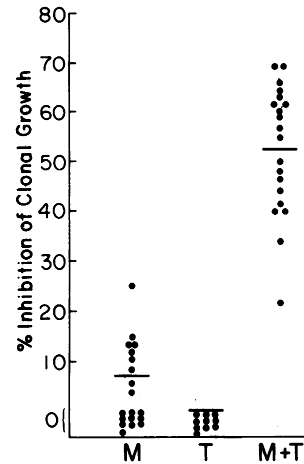


FIGURE 4 The effect of lactoferrin (0.01 pM) on CSA production/release by monocytes, T cells, and monocytes plus T cells in two assay systems (CSA in LCM and spontaneous colony growth). Each point represents a mean value from three to five plates from a single normal volunteer. Inhibition of CSA production is expressed as percent control clonal growth. Lactoferrin had no effect on the minimal CSA production by T lymphocytes. Lactoferrin mediated inhibition of CSA production by (*a*) monocytes ranged from 0 to 25% ($n = 20$, mean $= 7\pm7$), (*b*) by T lymphocytes was nil ($n = 10$), and (*c*) by M + T range from 22 to 70% (mean±SD = 52±17). Inhibition of CSA production/release by lactoferrin is consistent and significantly greater ($P < 0.001$) when CSA-producing cells are M + T than when CSA-producing cells are either M or T alone.

ferrin was ineffective. However, when T4 positive and T8 positive cells were removed, clonal growth was enhanced (submaximally), and lactoferrin functioned as a potent inhibitor of clonal growth. Methylprednisolone exposure failed to alter the activity of T lymphocytes in these assays (data not shown).

In four limiting dilution experiments (the results of one are shown in Fig. 6), as few as 10^3 T lymphocytes added to 5×10^5 monocytes in feeder layers effected significant ($P < 0.01$) enhancement of clonal growth in the overlayered marrow cells. In the second, third, and fourth studies, 10^4 cells maximally enhanced clonal growth. In no study did lactoferrin exert an inhibitory effect on CSA production or release by monocytes unless T lymphocytes were added back to the feeder layer. The limiting dilution curve was complex but maximal at 10^4 cells/ml in studies 2–4. For example, in all three of the studies a decrease in CSA enhancement and in the lactoferrin permissiveness was noted when T cell dose exceeded 10^5/plate.

We tested the hypothesis that T lymphocytes produce factors that augment CSA production by mononuclear phagocytes. However, in four separate experiments wherein mononuclear phagocytes were incubated in autologous T lymphocyte-conditioned medium (no detectable CSA), overall production of CSA by monocytes did not increase (data not shown).

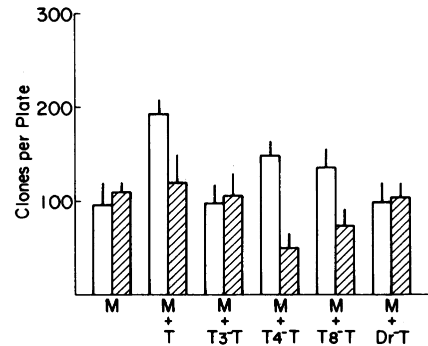


FIGURE 5 The effects of T lymphocytes and T lymphocyte subsets on CSA production by monocytes and on the ability of lactoferrin to inhibit CSA production by the mixture of cells. Bars and vertical lines represent mean±SD clones/plate in three to four replicate plates. In this one of six similar experiments 1.1×10^5 monocytes, 0.2×10^5 rabbit complement pretreated T lymphocytes and 1.0×10^5 T lymphocyte-depleted and phagocyte-depleted LDBMC were mixed in the spontaneous growth assay. All cells in the mix were autologous. In addition, 0.2×10^5 T cells were pretreated with complement and one of four monoclonal antibodies: OKT3, OKT4, OKT8, and E18/3 (Methods). T cells treated with OKT3 and complement are designated $T3^{-T}$ and so on. Note that whereas $T4^{-T}$ and $T8^{-T}$ consistently enhance CSA production and permit lactoferrin to inhibit CSA provision $T3^{-T}$ cells do not. Results were similar in two separate experiments that used different proportions of cells plated.

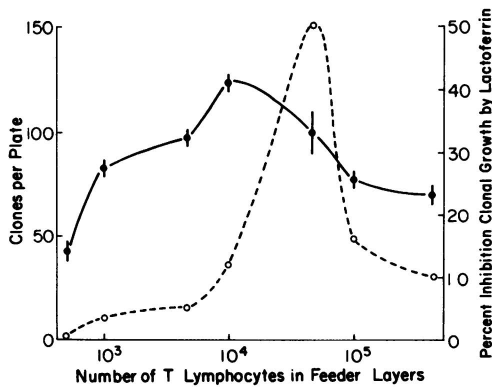


FIGURE 6 Limiting dilution curve. T cells of progressively increasing numbers were mixed with 5×10^5 monocytes in 0.5% agar medium feeder layers. Target cells in 1-ml methylcellulose cultures were 0.3×10^5 autologous T-depleted, phagocyte-depleted LDBMC/plate. Clones per plate (●) are expressed as mean±SD. Inhibition of clonal growth by lactoferrin (○) is expressed as percent inhibition; each point represents a mean of four to five replicate plates. Significant enhancement of clonal growth occurred when only 10^3 T lymphocytes were added. Furthermore, the inhibitory effect of lactoferrin on CSA production (expressed as percent control clonal growth) was noted only when T cells were added to the feeder layer. Identical experiments using cells from three other volunteers showed maximum enhancement of CSA production at 10^4 T cells and a maximum lactoferrin-mediated inhibition (48 and 55%) at 10^4 T cells. Both CSA-enhancing and lactoferrin-enhancing curves are nonlinear and show a high dose decrease, indicating that the regulatory interactions among monocytes, T lymphocytes, and lactoferrin are complex.

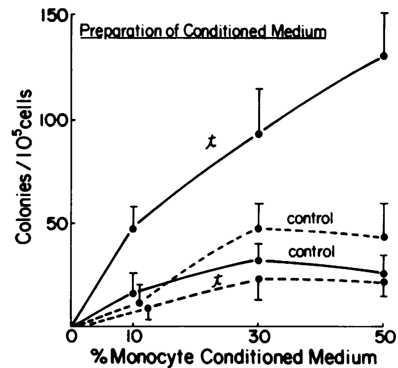


FIGURE 7 Monocytes produce a soluble factor that stimulates T lymphocytes to produce/release CSA. 10^5 T lymphocytes/ml were cultured for 5 d in 10, 30, and 50% monocyte-conditioned medium. The T lymphocyte-conditioned medium was then used (10% vol/vol), in a CSA assay, to stimulate clonal growth of autologous and allogeneic T-depleted, phagocyte-depleted LDBMC. This experiment is one of four, all of which showed similar results. Colonies per plate are expressed as mean±SD at each of three concentrations of monocyte-conditioned medium. "*t*" designates T lymphocyte cultures. "Control" represents monocyte-conditioned media to which no T lymphocytes had been added, incubated in parallel with the T lymphocyte cultures. Solid lines represent results obtained when the monocytes conditioned medium in the absence of lactoferrin. Dashed lines represent results obtained when monocytes conditioned medium in the presence of 10 fM lactoferrin. The results show that monocytes produce/release CSA (control, solid line), lactoferrin does not inhibit CSA production by monocytes (control, dashed lines), monocytes release a soluble factor that stimulates T-lymphocytes to produce CSA (*t*, solid line), and lactoferrin inhibits the production of this soluble factor (*t*, dashed line). Results in three other experiments were similar.

However, in four separate experiments when T lymphocytes were incubated in monocyte-conditioned media, CSA production (by T lymphocytes) increased (Fig. 7).

DISCUSSION

Thymus-dependent lymphocytes have been shown to exhibit a variety of functions in mammalian hematopoiesis. For example, T lymphocytes or their soluble products promote the growth of pluripotent (24–27) and committed (4, 28–30) stem cells. Mononuclear phagocytes share some of these functional characteristics. For example, human monocytes and macrophages can stimulate clonal proliferation of human erythroid progenitor cells by elaborating burst-promoting activity (31, 32) and can stimulate granulocyte-monocyte progenitor cells by producing or releasing CSA (2, 3, 5, 33). In fact, the monocyte-macrophage has been conventionally viewed as a primary source of CSA in vivo. More recently, however, using techniques that effectively separate monocytes from nonmonocytes, it has been noted that T lymphocytes and monocytes cultured together in the presence of antigen produce much more CSA than T lymphocytes or monocytes cultured alone (6, 7).

Using three assay techniques described above, we have confirmed that monocytes and T lymphocytes interact in CSA production (or release) even in the absence of antigen. More specifically, CSA production or release by monocytes plus either nonmonocytes (Fig. 1) or T lymphocytes (Figs. 2–6) was always greater than the sum of CSA production by either monocytes or T lymphocytes alone. In addition, the monocyte/T lymphocyte interaction does not seem to be genetically restricted (Fig. 3) and involves T3 and DR-positive cells (16). T lymphocytes that were treated with cytotoxic monoclonal antibodies defining either the human inducer/helper subclass OKT4 or human suppressor/cytotoxic T subclass OKT8, were capable of augmenting CSA production by monocytes (Fig. 5). However, we have not yet treated T cells with both OKT4 and OKT8 and, therefore, do not yet know whether the CSA augmentor T cells are homogeneous and all non-T4 and non-T8, or heterogeneous and partly T4 and/or partly T8.

Limiting dilution curves (Fig. 6) have consistently shown that the enhancement of CSA production is maximal when 10^3–5×10^4 lymphocytes are cultured with 5×10^5 monocytes and declines with increasing T cell numbers, a phenomenon recently reported in a mitogen-primed system (34). The optimal lymphocyte: monocyte ratio in our assays ranges from 1:50 to 1:10.

The mechanisms that regulate steady-state granulopoiesis in vivo are not at all understood. Largely based on the strength of in vitro work by Broxmeyer and his colleagues (9–11), lactoferrin is a candidate-regulatory molecule which, in vitro, has been reported to be a potent inhibitor of CSA production by mononuclear phagocytes. The proposal that lactoferrin may be a biologically relevant regulator of CSA production has been viewed with some skepticism by some investigators (35) who suggest that endogenous (e.g., FCS) lactoferrin levels exceed the reported threshold levels of 0.01 fM, express concern that maximum inhibition of CSA production by lactoferrin is not >50%, and point out that the findings of Broxmeyer et al. have not been confirmed in other laboratories. In our laboratory, using lactoferrin-depleted FCS, we confirmed that low doses of lactoferrin (0.01 nM–0.01 fM) inhibit by 40–60% the production/release of CSA by heterogeneous populations of leukocytes in vitro (12). However, we have failed to find that lactoferrin inhibits CSA production by mononuclear phagocytes from either the marrow or peripheral blood unless some T lymphocytes are added back to the monocytes in suspension (Figs. 2–6). The lymphocytes that permit the expression of lactoferrin's inhibitory effect, like those that augment CSA production, are all T3+ and DR+ but are not all T4+ or all T8+ (Fig. 5) and do not seem to function in a genetically restricted mode.

Although many details of the mechanism are unknown, we have found that lactoferrin completely inhibits the production of soluble factors by monocytes that stimulate T lymphocytes to produce CSA (Fig. 7). This observation explains why maximum CSA inhibition by lactoferrin ranges from 40 to 60%. Specifically, when lactoferrin, T lymphocytes, and monocytes are cultured together, lactoferrin effectively abrogates the contribution of the T lymphocytes to overall CSA production but has no effect on the production of CSA by monocytes (see Fig. 7). Our observations are in accord with other reports that lymphocytes do not bind lactoferrin, but monocytes do (36). The proposed mechanism of lactoferrin's effect is also in accord with the antigenic (Fig. 5) and functional (Fig. 6) similarities between the T lymphocytes that permit the expression of lactoferrin inhibitory activity and those that augment CSA production. In fact, on the strength of our observations we believe that these T lymphocyte activities are derived from the same subset. However, it should be recognized that, in our study, information on the antigenic nature of the interacting T cells was derived from cytotoxicity assays. We have not yet isolated these subsets for positive confirmatory studies. Therefore, we do not yet know whether important interactions occur between subsets.

Although the three clonal assays for colony-inhibiting activity are the same as those used in the pioneering studies of Broxmeyer et al. (9–11), a number of potentially important technical differences should be taken into account when comparing the results of this study with those of Broxmeyer's group. First, and of most importance, we have assiduously depleted T lymphocytes from both the CSA-producing cells and from the colony-forming target cells. Second, we avoided millipore filtration of conditioned media used for CSA assays because of the potential for loss of CSA activity on the filter (37). Third, we depleted lactoferrin from all FCS samples using the antibody affinity columns described above. Fourth, our human breast milk lactoferrin (20) may be somehow different from the lactoferrin preparations used in Broxmeyer's studies. For example, our lactoferrin loses its activity in high doses (Broxmeyer's preparations do not) because the molecule undergoes calcium-dependent and concentration-dependent polymerization in vitro and fails to exert inhibitory effects on CSA production when in a polymerized state (i.e., at high doses) (12). Under these conditions we have noted that human monocytes produce, as do some murine macrophages (38), a factor or factors that stimulate T lymphocytes to produce CSA in culture. Moreover, lactoferrin completely inhibits the production of these factors. Our observations indicate that, at least in vitro, monocytes and T lymphocytes exhibit important complex interactions in both positive and negative regulation of granulopoiesis.

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