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The role of monocyte cytotoxic factor (CF) in cytostasis mediated by IFN-γ-activated monocytes

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Summary. The role of monocyte cytotoxic factor (CF) in cytostasis mediated by lymphokine- and interferon gamma (IFN-γ)-activated monocytes was investigated. Recombinant IFN-γ as well as lymphokines enhanced monocyte-mediated cytostasis concomitantly with an increase in monocyte CF content. The incubation of lymphokines with monoclonal antibody against human IFN-γ inhibited cytostasis mediated by lymphokine-exposed monocytes, indicating that the main monocyte-activating factor in the lymphokine supernatant was IFN-γ. A neutralizing antiserum raised against purified CF inhibited monocyte-mediated cytostasis in a dose-dependent manner. When CF antiserum was added to monocytes previously exposed to lymphokines treated with a monoclonal antibody against IFN-γ, all cytostatic activity was completely abrogated. No inhibitory effect on cytostasis was observed with preimmune serum. The results indicate that CF is an effector molecule in cytostasis mediated by lymphokine- or IFN-γ-activated monocytes.

INTRODUCTION

Human monocytes activated with lymphokines acquire an increased capacity to induce cytolysis of various transformed cells *in vitro* (Hammerstrøm, 1979; Cameron & Churchill, 1979). Upon activation, monocytes also show enhanced production of a 40,000 MW cytotoxic protein factor (CF) which is found both as an extracellular soluble factor and as a monocyte plasma membrane-associated protein (Nissen-Meyer & Kildahl-Andersen, 1984; Kildahl-Andersen, Espevik & Nissen-Meyer, 1985b). CF appears to be an effector molecule in monocyte-mediated cytolysis, probably as a membrane-associated cytotoxin, since antiserum raised against purified CF inhibits monocyte-mediated cytolysis (T. Espevik, O. Kildahl-Andersen and J. Nissen-Meyer) submitted.

It is not clear whether mechanisms for monocyte-induced cytolysis and cytostasis are basically the same (Hamilton *et al.*, 1982). Monocyte–target cell contact appears to be involved in monocyte-mediated cytolysis (Espevik, Hammerstrøm & Halgunset, 1982), and this is in accordance with the notion that CF functions largely as a membrane-associated cytotoxin in cytolysis (Kildahl-Andersen *et al.*, 1985b). The cytostatic effect mediated by monocytes/macrophages may be reproduced using cell-free monocyte/macrophage supernatants (Stadecker *et al.*, 1977; Curry, 1978), suggesting that soluble factors participate in cytostasis and that cell-to-cell contact is not essential. In this paper, we have investigated the role of CF in cytostasis mediated by lymphokine- and IFN-γ-activated monocytes in order to determine whether CF is an effector molecule in this process, as it appears to be in monocyte-mediated cytolysis (Espevik *et al.*, submitted).

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MATERIALS AND METHODS

Materials

Human sera were obtained from the Blood Bank, University of Trondheim. Recombinant interferon gamma (IFN-γ) produced by Genentech Inc., San Francisco, CA, and monoclonal antibody GZ-4 against IFN-γ (6·5 mg protein/ml, 1 mg neutralizes at least 1000 U) were kindly supplied by Boehringer Ingelheim, Vienna, Austria. Monoclonal antibody B_3 against IFN-γ (500 neutralizing U/ml) was kindly provided by J. Vilcek and J. Le, New York, NY. Freeze-dried live bacillus Calmette-Guerin (BCG) was from Statens Seruminstitutt, Copenhagen, Denmark, and RPMI-1640 was from Gibco Biocult, Paisley, Renfrewshire, U.K. Anti-rabbit Ig ^{125}I-labelled species-specific $F(ab')_2$ fragment from donkey and [^{3}H]thymidine ([^{3}H]TdR) were obtained from the Radiochemical Centre, Amersham, Bucks, U.K.; Ficoll–Isopaque (Lymphoprep, specific weight 1077) from Nyegaard & Co., Oslo, Norway. Culture wells and dishes were purchased from Costar, Cambridge, MA, and microtitration plates were from Flow Laboratories, McLean, VA.

Isolation of human monocytes

Peripheral blood mononuclear cells were separated from venous blood of healthy adults by blood defibrination and centrifugation after layering on Ficoll–Isopaque. The mononuclear cells were washed twice in Hanks' balanced salt solution and suspended to a concentration of 4×10^6 cells per ml in human serum-supplemented medium (HSM) consisting of 25% pooled human AB Rh+ serum and 75% RPMI-1640 supplemented with glutamine (0·1 mM) and gentamicin (40 μg/ml).

Monocyte cultures

For cytostasis assay, 0·2 ml cell suspension was added to flat-bottomed microwells (well diameter 6 mm; Costar 3596) and, for quantification of CF in the monocytes, 1·5 ml cell suspension was added to culture wells with diameter of 35 mm (Costar 3506). After 90 min of incubation, the non-adherent mononuclear cells were aspirated, the monolayers washed three times with warm HBSS, and fresh HSM was added. After 1 and 3 days of *in vitro* culture, medium was changed. The cultures were incubated at 37° with 5% CO_2 in air with 100% humidity. The monolayers consisted of >95% phagocytosing monocytes on Day 4 of culture as judged by esterase staining, latex phagocytosis and phagocytosis of FITC-labelled *Candida albicans* (Hammerstrøm, 1981; Kildahl-Andersen & Nissen-Meyer, 1984).

Assay for monocyte-mediated cytostasis

The human non-adherent leukaemia cell line K-562 (Lozzio & Lozzio, 1975) was used for assaying monocyte-mediated cytostasis. Target cells (4×10^3) in HSM (0·1 ml) were added to monocytes activated for 24 hr with IFN-γ (1, 10, 100 and 1000 U/ml) or a 1:2 dilution of lymphokines prepared from human lymphocytes stimulated with BCG (Hammerstrøm, 1979). In some of the experiments, monocytes were activated for 24 hr with a 1:2 dilution of lymphokines preincubated for 2 hr at 37° with monoclonal antibodies against IFN-γ. On Day 4 of *in vitro* culture, activators and medium were removed and K-562 cells added to the monocytes. In some experiments, various amounts of rabbit preimmune serum or rabbit CF antiserum were added to the monocytes 15 min after start of coculture with the target cells. Target cell DNA synthesis was assayed after 24 hr of coculture by adding 1 μCi [^{3}H]TdR (specific activity 5 Ci/mmol) per well 4 hr before harvesting the cultures with a Titertek multiple-cell harvester. Inhibition of target cell [^{3}H]TdR incorporation (%TI) was calculated as:

$$\%TI = 100 - \frac{\text{c.p.m. (monocytes + K-562)} - \text{c.p.m. (monocytes)}}{\text{c.p.m. (K-562 plated alone)}} \times 100.$$

Compared to cytostasis, only modest cytolysis is registered after 24 hr coculture using K-562 as target cells (Espevik *et al.*, 1982).

Production of CF antiserum

Purification of CF, immunization with the purified CF antigen, and tests for the biological activity of the raised CF antiserum were performed as described earlier (Kildahl-Andersen & Nissen-Meyer, 1985).

Determination of CF content in the monocytes

Quantification of CF in non-activated and IFN-γ-activated monocytes was performed by a radioimmunoassay technique developed by modifying the enzyme-linked immunosorbent assay procedure described by Kenny & Dunsmoor (1983). The monocytes were detached from the culture dishes using a rubber policeman, counted, and lysed with 0·05% SDS and diluted in 50 mM carbonate buffer, pH 9·6 (coating buffer) to 100 cells/ml, of which 100 μl was added to

each well in Linbro/Titertek E.I.A. microtitration plates. After overnight incubation at room temperature, the plates were washed three times with PBS supplemented with 0·05% Tween 20 (PBS-Tween). Preimmune serum or CF antiserum, 1 μl in 100 μl PBS-Tween, was then added to each well and, after incubation for 90 min, the plates were again thoroughly washed in PBS-Tween. Anti-rabbit Ig, ^{125}I-labelled species-specific F(ab′)$_2$ fragment from donkey (specific activity 500–2000 Ci/mmol), 2 μl in 100 μl PBS-Tween, was then added to each well and the plates were again incubated for 90 min. After incubation and washing with PBS-Tween, 200 μl of 1% SDS were added to the wells to release the bound radioactive anti-rabbit Ig. After aspirating the content from each well, the radioactivity released was determined by gamma radiation counting.

Statistics

Results are given as the mean ± SD of triplicate or quadruplicate determinations in single experiments, or as the mean ± SEM of n separate experiments. P values were calculated by Student's t-test.

RESULTS

Effect of IFN-γ on monocyte-mediated cytostasis

The effect of activating monocytes with IFN-γ on monocyte-mediated cytostasis was determined. Monocytes incubated with various concentrations of IFN-γ exerted monocyte-mediated cytostasis in a dose-dependent manner (Fig. 1). Activating the monocytes with 100 U/ml IFN-γ increased cytostasis from 31% (non-activated monocytes) to 85% ($P < 0{\cdot}05$ for $n = 6$), indicating that IFN-γ alone is sufficient for activating the monocytes for cell-mediated cytostasis. Lymphokine-activated monocytes also exerted enhanced cytotstasis, corresponding to results obtained with monocytes activated with 10–100 U/ml IFN-γ (data not shown).

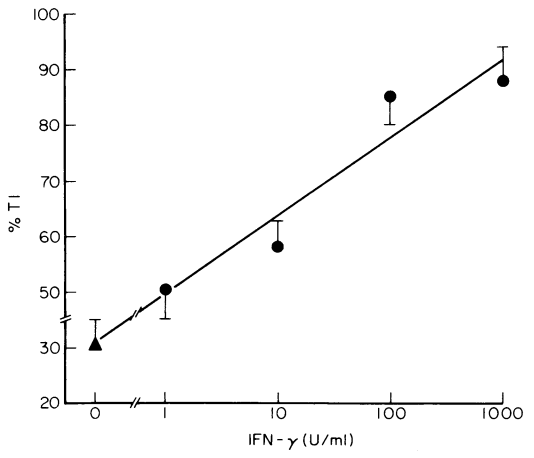

Figure 1. Effect of IFN-γ activation on monocyte-mediated cytostasis. Monocytes cultured *in vitro* for 3 days were activated 24 hr with IFN-γ after which the activator was removed and K-562 cells were added (●——●). Monocyte-mediated cytostasis was measured in a 24-hr assay; (▲) cytostasis obtained with non-activated monocytes. One of six similar experiments is presented.

Determination of relative CF content in IFN-γ-activated monocytes

The relative amount of CF in non-activated and IFN-γ-activated monocytes was determined by radioimmunoassay. Activating the monocytes for 24 hr with 10–1000 U/ml IFN-γ increased the amount of monocyte-associated CF in a dose-dependent manner (Table 1). Non-activated monocytes bound only 1/3 the amount of antiserum bound by monocytes activated with 1000 U/ml IFN-γ (Table 1).

Inhibition of lymphokine activation of monocytes for cytostasis by monoclonal antibodies against IFN-γ

In order to determine whether the IFN-γ in our lymphokine preparation was responsible for lymphokine activation of monocytes for increased cytostasis, the effect of monoclonal antibodies against IFN-γ on lymphokine activation was investigated. Lymphokines were incubated with various amounts of monoclonal antibodies, after which monocytes were

Table 1. Relative amounts of CF antiserum bound to monocytes activated with various concentrations IFN-γ

Amount IFN-γ (U/ml)	CF antiserum bound (c.p.m.*)
0	287 ± 2
10	446 ± 10
100	584 ± 98
1000	778 ± 54

* Measured as c.p.m. of anti-rabbit Ig, ^{125}I-labelled species-specific F(ab′)$_2$ fragment. Radioactivity bound to wells containing CF antiserum but no cells (240 ± 22 c.p.m.) has been subtracted. Wells containing preserum and cells bound 100 ± 33 c.p.m. Mean ± SEM of two experiments.

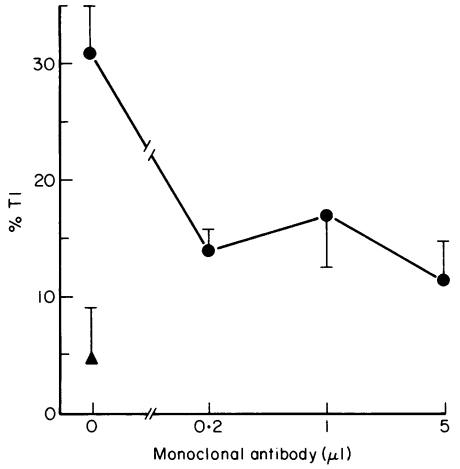

Figure 2. Inhibition of lymphokine activation of monocytes by monoclonal antibodies against IFN-γ. Lymphokines were incubated for 2 hr at 37° with various amounts of monoclonal antibodies (GZ-4) against IFN-γ, and were subsequently tested for their ability to activate monocytes for cell-mediated cytostasis (●——●); (▲) cytostasis obtained with non-activated monocytes. Monocyte activation and assay for cell-mediated cytostasis was performed as described in Fig. 1. One of four similar experiments is presented.

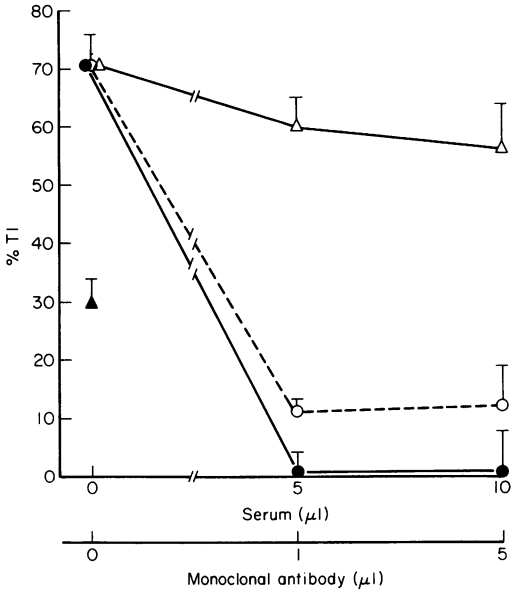

Figure 3. Inhibition of lymphokine-induced cytostasis by various amounts of CF antiserum (○----○), 10 μl CF antiserum and various amounts of monoclonal antibodies (B_3) against IFN-γ (●——●), and various amounts of preimmune serum (△—△); (▲) cytostasis obtained with non-activated monocytes. B_3 and lymphokines were incubated and tested for activation as described in Fig. 2. After removing the activator, K-562 cells were added and, 15 min later, CF antiserum or preimmune serum was added. Assay for monocyte-mediated cytostasis was performed as described in Fig. 1. One of five similar experiments is presented.

exposed for 24 hr to the untreated and antibody-treated lymphokines. Monoclonal antibody GZ-4 against IFN-γ reduced the lymphokine-induced monocyte-mediated cytostasis in a dose-related manner (Fig. 2). Similar results were also obtained with the monoclonal antibody B_3 against IFN-γ (data not shown). Lymphokine preparation treated with 5 μl monoclonal antibody reduced cytostasis mediated by the lymphokine-exposed monocytes from 31 to 11% ($P < 0{\cdot}05$ for $n = 4$), whereas non-exposed monocytes exerted 5% cytostasis (Fig. 2). The results show that the presence of IFN-γ in our lymphokine preparation was necessary for activation of monocytes for cell-mediated cytostasis. However, in none of our experiments was lymphokine-induced monocyte-mediated cytostasis reduced to the cytostasis exerted by non-activated monocytes.

The role of CF in cytostasis

In order to determine the role of CF in monocyte-mediated cytostasis, monocyte monolayers were exposed to lymphokines or lymphokines incubated with monoclonal antibody B_3 against IFN-γ, and at start of coculture with target cells, CF antiserum or preimmune serum was added to the cultures. CF antiserum, which neutralizes the cytotoxic activity of CF (Kildahl-Andersen & Nissen-Meyer, 1985), inhibited monocyte-mediated cytostasis in a dose-dependent manner (Fig. 3). With 10 μl CF antiserum present, lymphokine-induced cytostasis was reduced from 71 to 13% ($P < 0{\cdot}05$ for $n = 5$), which is markedly lower than the cytostasis exerted by non-activated monocytes (30%). When CF antiserum was added to monocyte monolayers previously exposed to lymphokines treated with monoclonal antibodies against IFN-γ, the cytostatic activity was completely abolished (Fig. 3) in all experiments ($P < 0{\cdot}02$). This inhibition was apparently not due to toxic proteins present in the rabbit serum, since preimmune serum did not inhibit monocyte-mediated cytostasis to any extent (Fig. 3). The results indicate that CF is an effector molecule in cytostasis mediated by lymphokine- or IFN-γ-activated monocytes.

DISCUSSION

Human monocytes activated with lymphokines acquire an increased capacity to induce cytolysis of various transformed cells *in vitro* (Hammerstrøm, 1979, 1981; Cameron & Churchill, 1979), and they show an increased production of a 40,000 MW cytotoxic protein factor (CF) (Nissen-Meyer & Hammerstrøm, 1982; Nissen-Meyer & Kildahl-Andersen, 1984). IFN-γ appears to be the main monocyte activator in lymphokine preparations responsible for increased cytolytic activity (Le & Vilcek, 1984; Spitalny & Havell, 1984) and CF production (Kildahl-Andersen *et al.*, 1985b). Upon monocyte activation, CF may accumulate both as a monocyte-released extracellular factor and as a protein on the monocyte plasma membrane (Kildahl-Andersen *et al.*, 1985b). CF appears to be involved in monocyte-mediated cytolysis, probably as a membrane-associated protein, since antiserum raised against purified CF inhibits monocyte-mediated cytolysis (Espevik *et al.*, submitted). Moreover, target cells which become resistant to the cytotoxic activity of CF upon a long-time exposure to CF also acquire resistance to monocyte-induced cytolysis (to be published).

In this communication, we report that IFN-γ as well as lymphokines enhanced monocyte-mediated cytostasis concomitantly with an increase in the monocyte CF content. IFN-γ in the lymphokine preparation appeared to be the main component responsible for activating the monocytes for enhanced cytostasis, since incubating the lymphokines with a monoclonal antibody against IFN-γ greatly reduced the cytostasis mediated by the lymphokine-exposed monocytes. CF appears to be an effector molecule in cell-mediated cytostasis, since CF antiserum had an inhibitory effect on cytostasis induced by monocytes. The effector mechanism for monocyte-mediated cytostasis is thus completely analogous to what has been observed for monocyte-mediated cytolysis (Espevik *et al.*, submitted). CF seems to participate both in monocyte-mediated cytolysis and cytostasis of K-562 target cells, in cytolysis as a membrane-bound effector molecule requiring cell-to-cell contact (Espevik *et al.*, submitted). Although cytostasis probably also involves membrane-associated CF and cell-to-cell contact to a significant extent, CF as a released factor probably also contributes greatly to monocyte-mediated cytostasis, since CF as a soluble factor has significant cytostatic activity against K-562 cells (Hammerstrøm, 1982; Kildahl-Andersen & Nissen-Meyer, 1984). The cytolytic activity of CF in solution is detectable only after a long exposure of K-562 cells to CF (Kildahl-Andersen, Bakke & Nissen-Meyer, 1985a), or upon using target cells, such as WEHI 164 fibrosarcoma cells, which are more sensitive to the lytic activity of CF (R. Austgulen, J. Hammerstrøm, T. Espevik and J. Nissen-Meyer, submitted). This observation is in monocyte-induced cytolysis against K-562 cells to any extent, a 48–72 hr coculture period is necessary (Hammerstrøm, 1979; Kildahl-Andersen *et al.*, 1985b). Only modest cytolysis is registered after 24 hr (Espevik *et al.*, 1982). Nonetheless, as shown in this paper, a high cytostatic activity against K-562 cells was obtained already after 24 hr coculture. However, differences in non-activated monocyte-mediated cytostasis (TI = 5–31%) was observed from one experiment to another (Figs 1–3). This was probably due to variations among individual blood donors, as culture conditions were kept unchanged in the experiments.

The cytostasis obtained with lymphokine-activated monocytes was not entirely inhibited when lymphokines were first treated with a monoclonal antibody against IFN-γ. This suggests the possible presence of factors other than IFN-γ in lymphokine supernatants which may cause release of CF to the medium, sufficient for inducing some cytostasis of K-562 cells but insufficient for inducing cytolysis. A complete inhibition of monocyte-induced cytostasis was obtained when monocytes were first exposed to lymphokines treated with monoclonal antibody against IFN-γ and subsequently incubated with CF antiserum during coculture. The complete inhibition was probably due to the reduced amount of CF synthesized and accumulated as a result of neutralization of IFN-γ, and consequently the amount of CF antiserum added at the start of coculture was sufficient for neutralizing all of the cell-bound and released CF.

CF may be identical to a cytotoxin derived from lipopolysaccharide-exposed, freshly isolated monocytes, reported by Matthews (1981, 1982, 1983a, b). HL-60 cells induced to differentiate into monocyte-like cells with 1α,25 dihydroxy vitamin D_3 show staining with CF antiserum, in accordance with the fact that HL-60 cells also gain cytotoxic activity upon differentiation (T. Espevik, to be published). HL-60 cells differentiated with 4β-phorbol-12β-myristate-13α-acetate (PMA) produce a human tumour necrosis factor. Recently, the gene encoding for this factor has been cloned (Pennica *et al.*, 1984). CF may be identical to the cloned tumour necrosis factor, and this question remains the subject for further investigation.

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