[CANCER RESEARCH 48, 1808–1811, April 1, 1988]

Augmentation of Release of Cytotoxin from Murine Bone Marrow Macrophages by γ-Interferon

Takafumi Okutomi and Masatoshi Yamazaki[1]

Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, Japan

ABSTRACT

γ-Interferon (IFN-γ) was shown to amplify cytotoxin release from murine bone marrow-adherent macrophages in response to an animal lectin as a second stimulus. This stimulation of cytotoxin release was demonstrated both *in vitro* and *in vivo* and was maximal 2 h after treatment with IFN-γ. No change in expression of surface antigens was detected on bone marrow-adherent cells treated with IFN-γ. IFN-γ also augmented production of cytotoxin from bone marrow-adherent cells that had been cultured for several days. This cytotoxin was inhibited by rabbit anti-tumor necrosis factor serum and had a molecular weight of 70,000. These findings showed that both freshly isolated and cultured bone marrow macrophages could release cytotoxin and that cytotoxin production in response to a second stimulus was augmented by IFN-γ. The results also suggest that IFN-γ can activate all macrophages *in vivo*, irrespective of their states of maturation and differentiation.

INTRODUCTION

A variety of peritoneal effector cells, including T-lymphocytes (1), natural killer cells (2), lymphokine-activated killer cells (3), macrophages (4), and polymorphonuclear leukocytes (5), have been examined for cytotoxic reactivity against tumor cells. However, it is unknown at which stage of maturation these cells become cytotoxic or whether fresh bone marrow cells, which are precursors of peritoneal cells, show cytotoxicity against tumor cells. Therefore, we have focused attention on the cytotoxicity of bone marrow cells and found that fresh bone marrow cells from normal mice can lyse syngeneic tumor cells in the presence of appropriate ligands (6). Furthermore, we have shown that fresh bone marrow cells can release a cytotoxin upon stimulation with loach egg lectin and that the main cells responsible for cytotoxin release are apparently immature macrophages (7). This paper shows that cytotoxin release in response to a second stimulus was amplified by treating mouse bone marrow macrophages with murine recombinant IFN-γ[2] and that the production of cytotoxin was maximal 2 h after treatment with IFN-γ and diminished after that.

MATERIALS AND METHODS

Bone Marrow Macrophages. Inbred male C3H/He mice were obtained from Shizuoka Experimental Animal Farm (Shizuoka, Japan) and used at 8 wk of age. Bone marrow cells were collected from femoral shafts by flushing the marrow cavity with Hanks' balanced salt solution. The dispersed cells were filtered through nylon mesh, washed twice with cold phosphate-buffered saline, and suspended in RPMI medium with 5% heat-inactivated fetal calf serum. Cell viability was determined with 0.3% trypan blue, and nucleated cells were counted after staining with 1% Türk stain. About 70% of the bone marrow cells from normal mice were nucleated, and their viability was about 90%. Bone marrow cells (1.6×10^6/well) were incubated in flat-bottomed 96-well plates for 1 h at 37°C to allow them to adhere to the plates. Then the medium was removed, and the adherent cells were washed 3 times with warm medium. These adherent cells were used for cytotoxic factor production.

Cytotoxic Assay. The cytotoxic activity of culture supernatants was determined by assay of killing of L929 cells, as described by Ruff and Giford (8). Briefly, L929 cells (8×10^4/well) were incubated with various dilutions of samples and actinomycin D (1 μg/well) for 18 h in 96-well trays containing 200 μl of medium (Eagle's minimal essential medium supplemented with 5% fetal calf serum). The cells were then stained with 0.1% crystal violet for 15 min, washed with water, and treated with 100 μl of 0.5% sodium dodecyl sulfate, and the absorbance at 590 nm of the solubilized material was measured. The survival ratio was calculated as the ratio of the absorption of the test sample to that of the control. The dilution of the sample giving the half-survival ratio (50% effective dose) was determined from the dose-response curve. The cytotoxic activity (units) in each test plate was calculated as the ratio of the 50% effective dose of the culture supernatant to that of rabbit tumor necrosis serum, as described previously (9). The cytotoxic activity of this rabbit tumor necrosis serum was equivalent to 6×10^3 units/ml of recombinant human tumor necrosis factor (kindly provided by Asahi Chemicals, Inc., Tokyo, Japan).

Agents. Loach (*Misgurnus anguillicaudatus*) egg lectin was kindly provided by Dr. F. Sakakibara and Dr. H. Kawauchi. This lectin appeared homogeneous on polyacrylamide gel electrophoresis and was identified as a M_r 50,000 glycoprotein with an isoelectric point of pH 6.6 (10).

No contaminating lipopolysaccharide was detected in this lectin preparation (11). Murine recombinant IFN-γ was kindly provided by Toray Industries, Inc. (Tokyo, Japan). Rabbit anti-TNF serum was a gift from Dr. S. Natori (Faculty of Pharmaceutical Sciences, University of Tokyo, Japan) (12).

Antibodies. Antimacrophage monoclonal antibodies (B12, C14) were prepared as follows (13). An 8-wk-old Wistar rat was immunized with 7.5×10^6 glycogen- or *Bacillus Calmette-Guérin*- or elicited peritoneal cells from C3H/He mice. On Day 21, the rat received further injections of 5×10^6 cells i.v. and 5×10^5 cells i.p. Three days after the second immunization, a spleen cell suspension of the immunized rat was fused with P3UI mouse myeloma cells in a 4:1 ratio. The supernatants of growing hybrids were tested 10 to 14 days after fusion by complement-dependent cytotoxic assay. Hybrids that secreted antibody with selective reactivity for macrophages were cloned by limiting dilution. Antibodies were obtained from culture supernatants of selected hybridomas. B12 antibody reacts with resident peritoneal macrophages and those elicited in various ways but not with lymphocytes. C14 antibody reacts with resident peritoneal macrophages but not with peritoneal macrophages obtained after i.p. injection of *B. Calmette-Guérin* (13).

Anti-asialo-GM1 antibody was purchased from Wako Pure Chemical Industries (Osaka, Japan). Anti-I-A^k and anti-Mac-1 monoclonal antibodies were obtained from Cedarlane Laboratories (Hornby, Ontario, Canada) and Sera-lab (Sussex, England), respectively.

Column Chromatography. Gel filtration was carried out in a high-performance liquid chromatography system (LC-6A; Shimadzu) fitted with a 0.75- x 60-cm column of G3000 SW (Tokyo Soda Manufacturing Co.). Material was eluted with phosphate-buffered saline at a flow rate of 1 ml/min.

RESULTS

Effect of IFN-γ on Cytotoxin Release from Bone Marrow-adherent Cells. We first examined the ability of IFN-γ to amplify cytotoxin release from murine bone marrow-adherent cells in response to a second stimulus. For this, bone marrow-adherent cells were incubated *in vitro* with IFN-γ and stimulated

Received 8/10/87; revised 12/5/87; accepted 12/28/87.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed.

[2] The abbreviations used are: IFN-γ, γ-interferon; TNF, tumor necrosis factor.

with loach egg lectin, and then the cell-free culture media were tested for cytotoxic activity against L-929 cells. Fig. 1 shows that IFN-γ amplifies cytotoxin release from bone marrow-adherent cells in response to loach egg lectin. This effect was maximal 2 h after addition of IFN-γ and then gradually decreased. IFN-γ alone did not induce cytotoxin release from bone marrow-adherent cells. Fig. 2 shows that 0.1 unit/ml of IFN-γ fully amplified cytotoxin release from bone marrow-adherent cells.

Next, we injected IFN-γ i.v. to test whether it augmented cytotoxin release from bone marrow-adherent cells. As shown in Fig. 3, cytotoxin release from bone marrow-adherent cells was augmented by i.v. IFN-γ injection into mice. This effect was also maximal 2 h after i.v. IFN-γ injection. These results indicate that IFN-γ can augment bone marrow-adherent cells *in vivo* as well as *in vitro*.

To determine whether IFN-γ stimulated cultured macrophages as well as fresh macrophages from the bone marrow, we examined the effect of IFN-γ on cytotoxin release from bone marrow-adherent cells that had been cultured for several days. Fig. 4 shows that IFN-γ augmented production of cytotoxin from bone marrow-adherent cells that had been cultured for several days as well as from fresh bone marrow-adherent cells. Most of the cultured cells showed the characteristics for mature macrophages in terms of their morphology and phagocytic activities for carbon particles and sensitized erythrocytes. These

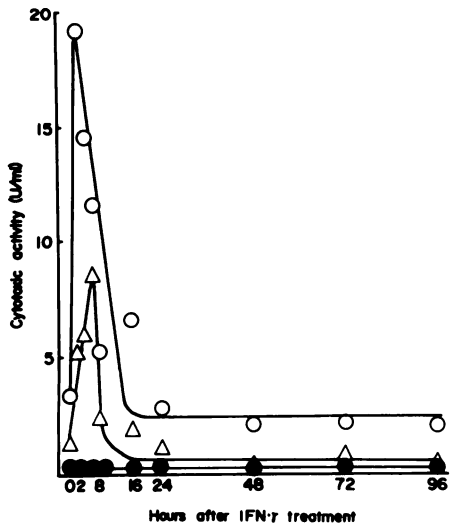

Fig. 1. Time course of cytotoxin release from IFN-γ-treated, bone marrow-adherent cells. Bone marrow-adherent cells with (○) or without (△) IFN-γ treatment (100 units/ml) were stimulated with loach egg lectin (20 μg/ml) for 2 h. Control cells were treated with IFN-γ (100 units/ml) without loach egg lectin (●).

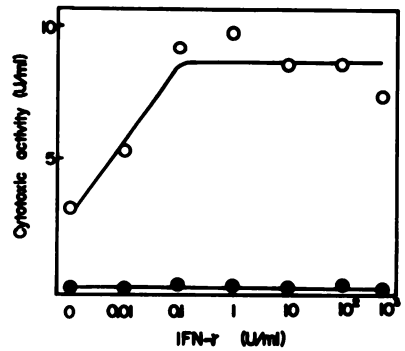

Fig. 2. Dose-response curve for IFN-γ. Bone marrow-adherent cells were treated with the indicated concentrations of IFN-γ for 4 h with (○) or without (●) stimulation with loach egg lectin (20 μg/ml) for 2 h.

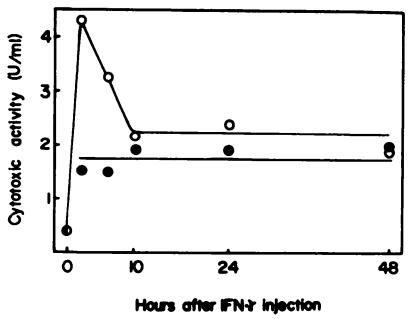

Fig. 3. Time course of cytotoxin release by bone marrow-adherent cells from IFN-γ-treated mice. Bone marrow-adherent cells were obtained from mice after i.v. injection of IFN-γ (10^4 units/mouse) (○) or saline (●) and stimulated with loach egg lectin (20 μg/ml).

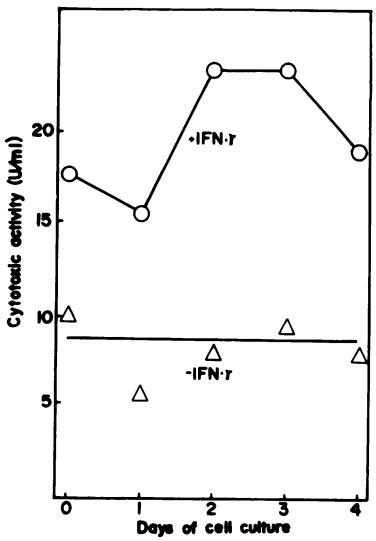

Fig. 4. Effects of IFN-γ treatment on cytotoxin release from cultured bone marrow-adherent cells. Bone marrow-adherent cells were cultured in 5% fetal calf serum:RPMI 1640 medium. After the indicated time, they were treated with (○) or without (△) IFN-γ (100 units/ml) for 4 h and then stimulated with loach egg lectin for 2 h.

results suggest that IFN-γ can augment cytotoxin release from both mature and immature macrophages.

Some Properties of Cytotoxin from IFN-γ-treated Bone Marrow-adherent Cells. We passed the culture supernatant from IFN-γ-treated, bone marrow-adherent cells through high-performance liquid chromatography column to determine the molecular weight of the cytotoxin. The cytotoxin was mainly eluted in a fraction corresponding to a molecular weight of about 70,000 (Fig. 5). Cytotoxin release from untreated bone marrow-adherent cells had a similar molecular weight (7).

Next, we examined the effect of rabbit anti-murine TNF serum on the cytotoxin released from IFN-γ-treated, bone marrow-adherent cells. As shown in Table 1, the cytotoxic activity in the culture medium of IFN-γ-treated, bone marrow-adherent cells was completely inhibited by anti-TNF serum.

Cells Responsible for Cytotoxin Release. We demonstrated that the release of cytotoxin from bone marrow-adherent cells was inhibited by anti-macrophage monoclonal antibody (B12) in the presence of complement, but not by anti-asialo-GM1 antibody (7). In an attempt to determine the antigens expressed on the surface of IFN-γ-treated, bone marrow-adherent cells, we tested the abilities of five antibodies to deplete cell subsets in the presence of complement. As shown in Fig. 6, the releases of cytotoxin from IFN-γ-treated and untreated bone marrow-adherent cells were inhibited by anti-macrophage monoclonal antibody (B12), but not by anti-asialo-GM1 antibody. C14

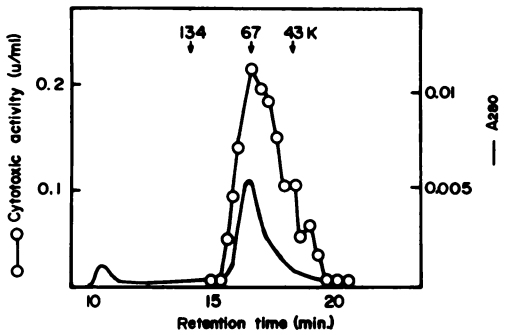

Fig. 5. Elution profile on high-performance liquid chromatography of cytotoxin from IFN-γ-treated, bone marrow-adherent cells. Bone marrow-adherent cells were treated with IFN-γ (100 units/ml) for 4 h and stimulated with loach egg lectin (20 μg/ml) for 2 h. The culture supernatants were subjected to high-performance liquid chromatography on a G3000SW column (0.7 x 65 cm). ↓, molecular weights of standards × 10^3.

Table 1 *Inhibition of bone marrow cytotoxin by rabbit anti-TNF serum*

Bone marrow-adherent cells were treated with IFN-γ (100 units/ml) for 4 h and stimulated with loach egg lectin (20 μg/ml) for 2 h. The culture supernatants were treated with rabbit anti-TNF serum or preimmune serume for 1 h on ice and assayed for cytotoxicity.

Treatment	Cytotoxic activity (units/ml)	Inhibition (%)
Preimmune serum	13.4	0
Anti-TNF serum	0.1	99.3

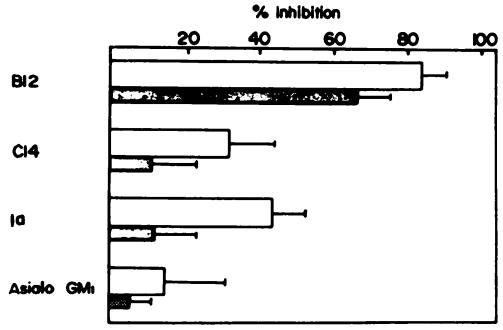

Fig. 6. Effects of various antibodies on cytotoxin release from bone marrow-adherent cells. Bone marrow-adherent cells after treatment with (□) or without (■) IFN-γ (100 units/ml) for 4 h were incubated with various antibodies (10-fold final dilution) for 30 min at 37°C and for 20 min at 37°C after addition of Low-Tox-M rabbit complement. Then the medium was removed, and the adherent cells were washed twice. These cells were stimulated with loach egg lectin (20 μg/ml), and the culture supernatants were harvested after 2 h.

monoclonal antibody, which reacts with resident peritoneal macrophages but does not activate peritoneal macrophages, partly inhibited cytotoxin release from IFN-γ-treated, bone marrow-adherent cells. The release of cytotoxin from IFN-γ-treated, bone marrow-adherent cells was also inhibited about 40% by anti-Ia monoclonal antibody. These results suggest that the adherent cells responsible for cytotoxin release may be of macrophage lineage.

DISCUSSION

In this work we found that IFN-γ amplified cytotoxin release from bone marrow-adherent cells in response to loach egg lectin as a second stimulus. The release of cytotoxin in response to loach egg lectin was maximal 2 h after treatment with IFN-γ (Figs. 1 and 3). Similar results were obtained when murine peritoneal macrophages were treated with IFN-γ (data now shown). Moreover, we showed that IFN-γ augmented cytotoxin release from bone marrow macrophages that had been cultured for several days as well as from freshly isolated bone marrow macrophages (Fig. 4). Therefore, we think that IFN-γ can activate all macrophages *in vivo*, irrespective of their states of maturation and differentiation.

There are reports of macrophage surface antigens whose presence or absence is correlated with activation of macrophages (14–19). We also reported that asialo-GM1 antigen is expressed on peritoneal macrophages that release cytotoxin (13). However, anti-asialo-GM1 antibody scarcely reacted with IFN-γ-treated, bone marrow-adherent cells. Moreover, IFN-γ-treated and untreated bone marrow-adherent cells showed similar expressions of surface antigens. These results suggest that augmentation of cytotoxin release by IFN-γ treatment was not correlated with expression of surface antigens on bone marrow macrophages. The cytotoxin released from both IFN-γ-treated and untreated bone marrow-adherent cells was eluted mainly in the fraction corresponding to a molecular weight of about 70,000. The molecular weight of TNF from peritoneal macrophages appeared to be about 55,000 by high-performance liquid chromatography (data not shown), but the cytotoxin released from IFN-γ-treated bone marrow macrophages was almost completely inhibition by anti-TNF serum. Therefore, the cytotoxin released from IFN-γ-treated bone marrow macrophages may be TNF or a TNF-like protein. We have reported that macrophages (20–23), polymorphonuclear leukocytes (24–26), and bone marrow cells (6) from mice lysed tumor cells when stimulated with certain plant and animal lectins. Furthermore, we have reported that these lectins could induce release of a cytotoxin from bone marrow cells (7), polymorphonuclear leukocytes (25), and macrophages (13, 27). These data suggest that both mature and immature phagocytes have the ability to release a TNF-like cytotoxin.

REFERENCES

1. Kaufman, Y., Berke, G., and Eshhar, Z. Cytolytic T-cell hybridomas. *In:* E. Pick (ed.), Lymphokines, Vol. 5, pp. 277–289. New York: Academic Press, Inc., 1982.
2. Herberman, R. B., and Ortaldo, J. R. Natural killer cells: their role in defenses against disease. Science (Wash. DC), *214:* 24–30, 1981.
3. Grimm, E. A., and Rosenberg, S. A. The human lymphokine-activated killer cell phenomenon. *In:* E. Pick (ed.), Lymphokines, Vol. 9, pp. 279–311. New York: Academic Press, Inc., 1984.
4. Adams, D. C., and Hamilton, T. A. The cell biology of macrophage activation. Annu. Rev. Immunol., *2:* 283–318, 1984.
5. Gerrad, T. L., Cohen, D. J., and Kaplan, A. M. Human neutrophil-mediated cytotoxicity to tumor cells. J. Natl. Cancer Inst., *66:* 483–488, 1981.
6. Nakajima-Masuko, Y., Okutomi, T., Abe, S., Mizuno, D., and Yamazaki, M. Presence in mouse bone marrow of mediator-dependent cytotoxic effector cells against tumors. Jpn. J. Exp. Med., *53:* 227–233, 1983.
7. Okutomi, T., Nakajima, Y., Sakakibara, F., Kawauchi, H., and Yamazaki, M. Induction of release of cytotoxin from murine bone marrow cells by an animal lectin. Cancer Res., *47:* 47–50, 1987.
8. Ruff, M. R., and Gifford, G. E. Tumor necrosis factor. *In:* E. Pick (ed.), Lymphokines, Vol. 2, pp. 235–272. New York: Academic Press, Inc., 1981.
9. Satoh, M., Inagawa, H., Minagawa, H., Kajikawa, T., Oshima, H., Abe, S., Yamazaki, M., and Mizuno, D. Endogenous production of TNF in mice long after BCG sensitization. J. Biol. Response Mod., *5:* 117–123, 1986.
10. Sakakibara, F., Takayanagi, G., and Kawauchi, H. An L-rhamnose-binding lectin in the egg of *Misgurnus anguilicaudatus.* J. Pharm. Soc. Jpn., *101:* 918–925, 1981.
11. Nakajima, Y., Suzuki, H., Sakakibara, F., Kawauchi, H., Mizuno, D., and Yamazaki, M. Induction of a cytotoxin from murine macrophages by an animal lectin. Jpn. J. Exp. Med., *56:* 19–25, 1986.
12. Itoh, A., Ohsawa, F., Ohkuma, Y., and Natori, S. Participation of tumor killing factor in the antitumor effect of *Sarcophaga* lectin. FEBS, *201:* 37–40, 1986.
13. Okutomi, T., Satoh, M., Oshima, H., and Yamazaki, M. Antigens associated with various cytotoxic activities of murine peritoneal macrophages. Microbiol. Immunol., *30:* 1037–1047, 1986.
14. Taniyama, T., and Tokunaga, T. Monoclonal antibodies directed against mouse macrophages in different stages of activation for tumor cytotoxicity. J. Immunol., *131:* 1032–1037, 1983.
15. Koestler, T. P., Riemen, D., Muirhead, K., Greig, R. G., and Pote, G. Identification and characterization of a monoclonal antibody to an antigen

expressed on activated macrophages. Proc. Natl. Acad. Sci. USA, *81:* 4505–4509, 1984.
16. Bernard, A. F., and Howard, R. P. Characterization of a monoclonal antibody defining a macrophage activation specific cell surface antigen. Mol. Immunol., *21:* 681–684, 1984.
17. Someya, A. Specific surface antigens expressed on activated mouse peritoneal macrophages and recognized by a novel monoclonal antibody. Immunology, *56:* 683–688, 1985.
18. Mentzer, S. J., Faller, D. V., and Burakoff, S. J. Interferon-γ induction of LFA-1-mediated homotypic adhesion of human monocytes. J. Immunol., *137:* 108–113, 1986.
19. Ezekouitz, R. A. B., and Gordon, S. Down-regulation of mannosyl receptor-mediated endocytolysis and antigen F4/80 in *Bacillus Calmette-Guérin*-activated mouse macrophages. Role of T-lymphocytes and lymphokines. J. Exp. Med., *155:* 1623–1637, 1982.
20. Kurisu, M., Yamazaki, M., and Mizuno, D. Induction of macrophage-mediated tumor lysis by the lectin wheat germ agglutinin. Cancer Res., *40:* 3798–3803, 1980.
21. Yamazaki, M., Esumi-Kurisu, M., Mizuno, D., Ogata, K., and Kamiya, H. Marine animal lectin-dependent tumor recognition by macrophages. Gann, *74:* 405–411, 1983.
22. Yamazaki, M., Okutomi, T., Sakakibara, F., Kawauchi, H., and Mizuno, D. Macrophage-mediated tumor lysis induced by loach egg lectin. J. Leukocyte Biol., *35:* 241–250, 1984.
23. Nakajima, H., Komano, H., Esumi, M., Abe, S., Yamazaki, M., Natori, S., and Mizuno, D. Induction of macrophage-mediated tumor lysis by an animal lectin, *Sarcophaga perigrina* agglutinin. Gann, *73:* 627–632, 1982.
24. Tsunawaki, S., Oshima, H., Mizuno, D., and Yamazaki, M. Induction of polymorphonuclear leukocyte-mediated cytolysis by wheat germ agglutinin and antitumor antibody. Gann, *74:* 264–270, 1983.
25. Ikenami, M., and Yamazaki, M. Participation of polymorphonuclear leukocyte-derived factor in murine tumor cell killing. Br. J. Cancer, *52:* 575–581, 1985.
26. Yamazaki, M., Ikenami, M., Komano, H., Tsunawaki, S., Kamiya, H., Natori, S., and Mizuno, D. Polymorphonuclear leukocyte-mediated cytolysis induced by animal lectin. Gann, *74:* 576–583, 1983.
27. Kajikawa, T., Nakajima, Y., Hirabayashi, J., Kasai, K., and Yamazaki, M. Release of cytotoxin by macrophages on treatment with human placenta lectin. Life Sci., *39:* 1177–1181, 1986.