Induction of Release of Cytotoxin from Murine Bone Marrow Cells by an Animal Lectin

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ABSTRACT

An animal lectin purified from loach (Misgurnus anguillicaudatus) eggs induced release of cytotoxin from fresh bone marrow cells from mice, although the other lectins tested, wheat germ agglutinin, concanavalin A, and phytohemagglutinin did not. The cytotoxin released from bone marrow cells was a heat-labile protein with a molecular weight of 70,000. The main cells responsible for release of M, 70,000 cytotoxin seemed to be of macrophage lineage, since they adhered to plastic and were sensitive to certain antibodies for markers of macrophages. However, they did not express asialo GMI antigen which is expressed by activated macrophages. Removal of cells that phagocytized iron did not diminish but rather enhanced the release of cytotoxin. Therefore, active bone marrow cells appeared to be immature, not mature macrophages. These data suggest that immature bone marrow cells that are not specifically activated have a cytolytic potency against tumor cells and that internal animal lectins may induce release of the cytotoxin from these cells.

INTRODUCTION

Studies on cytotoxic effector cells against tumor cells have been focused on peripheral cells such as cytotoxic T-lymphocytes (1), natural killer cells (2), lymphokine-activated killer cells (3), macrophages (4), and polymorphonuclear leukocytes (5). These cells are all known to show cytoidal activity against tumor cells in vitro. However, it is unknown at which stage of maturation these cells become cytotoxic or whether fresh bone marrow cells, which are precursors of peripheral cells, show cytotoxicity against tumor cells. Therefore, we have focused attention on the cytotoxicity of bone marrow cells, namely central cells, and found that fresh bone marrow cells from normal mice can lyse syngeneic tumor cells in the presence of appropriate ligands (6). In this study, in an attempt to determine how fresh bone marrow cells cause cytolsis, we examined whether a soluble cytotoxin participated in tumor killing. This paper shows that bone marrow cells released a cytotoxin in the presence of an animal lectin and that the cells responsible for its release may be immature macrophages.

MATERIALS AND METHODS

Bone Marrow Cells. Inbred male C3H/He mice were obtained from Shizuoka Experimental Animal Farm (Shizuoka, Japan) and used at 8 weeks 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 with two changes of cold phosphate-buffered saline and suspended in RPMI 1640 medium with 10% heat-inactivated fetal calf serum. Cell viability was counted with 0.3% trypan blue and nucleated cells were counted with a hemocytometer. 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.

Cytoxic Assay. The cytotoxic activity of culture supernatants was determined by assay of killing of L929 cells, as described by Ruff and Gifford (7). Briefly, L929 cells (8 × 10^5/well) were incubated with various dilutions of sample and actinomycin D (0.2 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). Then the cells were stained with 0.1% crystal violet for 15 min, washed with water, and treated with 100 µl of 0.5% sodium dodecyl sulfate. 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 culture to that of the control without sample. The dilution of the sample giving the half-survival ratio (50% effective dose) was obtained from a 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 (8). The cytotoxic activity of this rabbit tumor necrosis serum was equivalent to recombinant human tumor necrosis factor, 6 × 10^4 units/ml (kindly provided by Asahi Chem. Ind., Tokyo, Japan).

Antigens. Loach (Misgurnus anguillicaudatus) egg lectin was purified by chromatography on two types of ion-exchange resin and affinity chromatography on L-rhamnose coupled with epoxy-activated Sepharose 6B. This lectin appeared homogeneous on polyacrylamide gel electrophoresis and was identified as a glycoprotein of Mr, 50,000 with an isoelectric point of pH 6.6 (9). No contaminating I I'S' was detected by chromatography on two types of ion-exchange resin and affinity chromatography on L-rhamnose coupled with epoxy-activated Sepharose 6B. This lectin appeared homogeneous on polyacrylamide gel electrophoresis and was identified as a glycoprotein of Mr, 50,000 with an isoelectric point of pH 6.6 (9). No contaminating I I'S' was detected by chromatography on two types of ion-exchange resin and affinity chromatography on L-rhamnose coupled with epoxy-activated Sepharose 6B. This lectin appeared homogeneous on polyacrylamide gel electrophoresis and was identified as a glycoprotein of Mr, 50,000 with an isoelectric point of pH 6.6 (9). No contaminating I I'S' was detected by chromatography on two types of ion-exchange resin and affinity chromatography on L-rhamnose coupled with epoxy-activated Sepharose 6B. This lectin appeared homogeneous on polyacrylamide gel electrophoresis and was identified as a glycoprotein of Mr, 50,000 with an isoelectric point of pH 6.6 (9). No contaminating I I'S' was detected.
to induce cytotoxin release from bone marrow cells of mice was examined. LPS was used as a control. Bone marrow cells from normal mice were incubated in vitro with various lectins or LPS, and then the cell-free culture media were tested for cytotoxic activity against L929 cells. Fig. 1 shows that LPS and loach egg lectin induced cytotoxin release from bone marrow cells into the medium, whereas wheat germ agglutinin, concanavalin A, and phytohemagglutinin did not. Culture supernatants from bone marrow cells alone did not show any cytotoxicity. Loach egg lectin alone at below 100 µg/ml also had no cytotoxicity against L929 cells.

Next, we examined the dose response of loach egg lectin and the kinetics of cytotoxin release. Loach egg lectin induced concentration-dependent cytotoxin release from bone marrow cells (Fig. 2). Fig. 3 shows that the cytotoxin activity induced by loach egg lectin reached a peak after 3 h and then decreased. Thus the release of cytotoxin was transient and the cytotoxin was unstable.

Some Properties of Cytotoxin from Bone Marrow Cells Induced by Loach Egg Lectin. The temperature stability of a crude preparation of the cytotoxin released from bone marrow cells was examined. As shown in Table 1, the cytotoxin was heat labile, losing about 90% of its activity in 15 min at 70°C. The cytotoxin also lost its activity on treatment with trypsin.

Next, we passed the culture supernatant through a highperformance liquid chromatography column to estimate the molecular weight of the cytotoxin. The cytotoxin induced by loach egg lectin was mainly eluted in the fraction corresponding to a molecular weight of about 70,000 (Fig. 4).

Cells Responsible for Cytotoxin Release. Bone marrow cells consist of many types of cells, such as erythrocytes, polymorphonuclear leukocytes, monocytes, lymphocytes, and their immature cells. To determine which types of cells release cytotoxin, we separated bone marrow cells into plastic-adherent and nonadherent cells. Fig. 5 shows that both adherent and nonadherent bone marrow cells released cytotoxin in the presence of loach egg lectin but that the release by adherent cells was several times greater than that by nonadherent cells in terms of activity per cell (Fig. 5, □). Therefore, the activity of the nonadherent fraction may be ascribed to contaminating adherent cells in it. Adherent cells (about 30% of the bone marrow cells) consist of morphologically heterologous populations, but with few typical monocytes/macrophages (less than 10% of the adherent cells).

In an attempt to determine the antigens expressed on the surface of adherent cells, the ability of five antibodies to detect surface antigens were tested in the presence of complement. As shown in Fig. 6, the release of cytotoxin from adherent cells...
The culture supernatants were harvested after 2 h and assayed for cytotoxicity. Adherent and nonadherent cells were stimulated with loach egg lectin (20 μg/ml). These cells were washed twice. These cells were stimulated with loach egg lectin (20 μg/ml) and the culture supernatants were harvested after 2 h.

Cytoxic activity (units/ml) vs Inhibition (%) of low-tox-M rabbit complement. Then the medium was removed and the adherent bone marrow cells. Adherent bone marrow cells 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. GMI, GM1, Anti-Mφs (B12), Anti-Mac-1, Anti-la, and Anti-asialo GM1 were assayed for cytotoxicity.

Fig. 6. Effects of various antibodies on cytotoxin release from adherent bone marrow cells. Adherent bone marrow cells 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. GMI, GM1.

Fig. 7. Effect of iron powder treatment on cytotoxin release from bone marrow cells. Bone marrow cells (8 × 10⁶ cells/ml) were incubated with (a–d) or without (e) iron powder (8 × 10⁶/ml) for 1 h at 37°C and then iron-phagocytized cells (c) were obtained by magnet. Residual cells did not phagocytize iron powder (d). Then the cells were adjusted to 8 × 10⁶ cells/ml and incubated with loach egg lectin (20 μg/ml). The culture supernatants were harvested after 2 h and assayed for cytotoxicity.

was inhibited about 80% by anti-Mac-1 monoclonal antibody (B12) and about 50% by anti-Mac-1 antibody. Anti-la antibody caused different extents of inhibition in four experiments, and so its effect was not clear. Anti-asialo GM1 antibody did not inhibit cytotoxin release. These results suggest that the adherent cells responsible for cytotoxin release may be of macrophage lineage.

Next, we examined whether mature phagocytes could release the cytotoxin. As shown in Fig. 7, removal of cells that phagocytized iron did not diminish but rather enhanced the release.

Therefore, mature phagocytes, which phagocytize iron powder, are not the main cells in bone marrow responsible for cytotoxin release and may in fact cause inhibitory control of the active cells.

DISCUSSION

We have shown that fresh bone marrow cells can release a cytotoxin on stimulation with loach egg lectin as well as with LPS. Loach egg lectin was similar to LPS in its ability to induce cytotoxin, but we have shown that it does not contain LPS (10). Therefore, the induction of cytotoxin release by loach egg lectin is not due to contaminating LPS. This cytotoxin is rather unstable, since cytotoxic activity was observed within 5 h after stimulation and disappeared within 15 h (Fig. 3).

The main cells responsible for cytotoxin release seemed to be of macrophage lineage, since they adhered to plastic (Fig. 5), and their ability to release cytotoxin was lost when they were treated with antimacrophage or anti-Mac-1 antibodies (Fig. 6). However, these cells did not express asialo GM1 antigen (Fig. 6), which is expressed by activated macrophages such as BCG-elicited macrophages (11). Moreover, these cells did not show phagocytic activity (Fig. 7). These results suggest that the bone marrow cells that release the cytotoxin are immature and not mature macrophages. However, further studies are required to determine whether these cells are monoblasts, premonocytes, or monocytes.

Much attention has been paid to the role of peripheral cells in host defense. Most of the cells responsible for the host defense mechanisms are thought to originate in the bone marrow and to become effector cells after differentiation, maturation, and activation. But more immature cells were reported to be involved in defense against infection (12) and tumors (13) and in allograft rejection (14, 15). In this study we also found that bone marrow cells that were not already specially activated had cytotoxic activity like that of peripheral cells. The amount of cytotoxin released per adherent cell was equivalent to that released by BCG-elicited peritoneal macrophages (data not shown). We have reported that loach egg lectin could induce cytotoxin release from peritoneal macrophages only when they were activated (10). In contrast, immature bone marrow cells without activation have cytolytic potency against tumor cells. Since there is a large pool of bone marrow cells, their participation must be taken into account when considering the host defense system against tumors.

We reported that macrophages (16), polymorphonuclear leukocytes (17, 18), and bone marrow cells (6) from mice lyse tumor cells in the presence of a plant lectin (lectin-dependent cellular cytotoxicity). Some selected animal lectins could also induce lectin-dependent cellular cytotoxicity (19-22). Therefore, internal lectins may be involved in tumor recognition and destruction. Active lectins bind effector cells to tumor cells (23, 24) and at the same time stimulate effector cells to release cytotoxin (10). Thus induction of cytotoxin may be one role of internal lectins. We speculate that cytotoxic factors are produced in situ by internal lectins and are effective in the host defense mechanism.

REFERENCES


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