Granulocyte- and Granulocyte-Macrophage Colony-Stimulating Factors Enhance Neutrophil Cytotoxicity Toward HIV-Infected Cells

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Although the control of retroviral disease in animal systems often involves antibody-dependent cell-mediated cytotoxicity (ADCC), the role of cytotoxic function in human retroviral disorders is uncertain. The ability of the neutrophil to kill HIV-infected targets directed by antiviral antibody was examined. Neutrophils from patients with AIDS killed HIV-infected MOLT-3A cells in a manner equivalent to neutrophils obtained from normal volunteers. Both granulocyte- and granulocyte-macrophage colony-stimulating factors (G-CSF and GM-CSF) markedly augmented the cytotoxic function. Studies done with fractionated human antisera revealed that ADCC to HIV-infected cells was mediated only by antibody to the env glycoprotein. ADCC in this system was not dependent on oxidative metabolism because neutrophils from patients with chronic granulomatous disease (CGD) were capable of CSF-augmented cytotoxicity. Although ADCC can be mediated by various classes of lymphocytes and mononuclear phagocytes, such cells may be infected by HIV. Because the neutrophil apparently is not productively infected by the virus, it is an ideal cell to focus on with regard to cytotoxic function in AIDS patients. The findings regarding neutrophil ADCC in AIDS are clinically relevant because the availability of CSFs now permits therapeutic regulation of neutrophils in AIDS patients, and presumably natural antibody may be useful in targeting HIV-infected cells for neutrophil cytotoxicity in vivo.

HUMAN IMMUNODEFICIENCY virus (HIV) is the etiologic agent of AIDS. Although HIV infects T4 lymphocytes and impairs host defense mechanisms that rely on T4 lymphocyte cooperativity, the global compromise of host defense in AIDS relates importantly to quantitative and qualitative defects in other effector cell types, such as mononuclear phagocytes and neutrophils.

The cellular immune response against HIV has not been clearly elucidated; reports have generally focused on antibodies to HIV proteins that have been identified in the sera of most AIDS patients. Such antibodies may or may not be neutralizing, but all have the capacity to interact with effector cell Fc receptors in an antibody-dependent cell-mediated cytotoxicity (ADCC) reaction. In vivo and in vitro evidence suggests that ADCC may play a primary role in host defense against tumors and virus-infected cells. Recent reports have therefore attempted to demonstrate a role for ADCC in killing HIV-infected cells.

A number of different effector cells can mediate ADCC, including lymphocytes, natural killer (NK) cells, monocyte/macrophages, and neutrophils. Neutrophil ADCC is markedly augmented in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) or granulocyte colony-stimulating factor (G-CSF). We have also shown that AIDS patients' neutrophils function normally with respect to ADCC and their activity can be enhanced by GM-CSF in vivo and in vitro. Because neutrophils from AIDS patients appear not to be productively infected with HIV and their cytotoxic function can be increased, the neutrophil is an ideal effector cell to focus on with regard to cytotoxicity against HIV-infected targets. We report here that GM-CSF and G-CSF enhance AIDS patients' neutrophil cytotoxicity against HIV-infected target cells in vitro. This cytotoxicity is antibody-dependent and is most effectively mediated by antibodies recognizing the env protein of HIV.

MATERIALS AND METHODS

Purification of neutrophils from peripheral blood. Neutrophils were isolated from heparinized peripheral blood of patients and healthy volunteers by Ficoll-Hypaque gradient centrifugation, followed by dextran sedimentation, as previously described. The resulting cell preparation contained more than 97% viable neutrophils.

Recombinant GM-CSF and G-CSF. Purified GM-CSF produced in Chinese hamster ovary (CHO) cells was provided by the Genetics Institute, Inc (Cambridge, MA). Purified G-CSF expressed in Escherichia coli (unglycosylated) was provided by Sandoz, Inc (Hanover, NJ). Each of these two purified proteins migrated as single bands on SDS-PAGE, and the protein concentration was estimated by the Bio-Rad protein assay. Both proteins were functionally active and could be used interchangeably. Human G-CSF was provided by Drs S. Asano and F. Takaku. Both GM-CSF and G-CSF were diluted in endotoxin-free diluent (phosphate-buffered saline with 0.02% bovine serum albumin).

Virus strains and cell lines. The virus strain HTLV-III/B was propagated in MOLT-3A cells. Briefly, HIV infection of MOLT-3A was initiated by incubating 3 x 10⁶ MOLT-3A cells with 0.5 mL of a filtered, cell-free supernatant fluid harvested from an HTLV-III B-infected MOLT-4 cell culture (provided by Dr I. S. Y. Chen) for 2 hours at 37°C. Infecting inoculum (0.5 mL) was estimated at 30 to 50 ng of viral p24 protein. Control cells were mock-infected with a filtered, cell-free supernatant fluid from an uninfected MOLT-4 cell culture; 10 μg/mL of Polybrene (Sigma, St Louis) was added to both infecting and mock-infecting supernatant fluids. After virus adsorption the cells were pelleted and resuspended in fresh medium to a concentration of 1 x 10⁶ cells/mL. Filtered and cell-free supernatants were obtained from the infected and mock-infected MOLT-3A

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cultures and assayed at day 14 for p24 antigen release using an HIV antigen enzyme immunoassay (Abbott Laboratories, North Chicago).

Cell lines were Mycoplasma free and were maintained at 37°C in Iscove's modified Dulbecco's medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal calf serum (FCS), 1% glutamine, and antibiotics.

Neutrophil ADCC assay. Peripheral blood neutrophils were isolated from normal donors, AIDS patients, and patients with chronic granulomatous disease (CGD) and incubated with either 100 pmol/L GM-CSF or 500 pmol/L G-CSF for 90 minutes at 37°C. Neutrophil cytotoxicity was assayed as previously described using G-CSF- or GM-CSF-primed neutrophils or unprimed neutrophils as effectors and 3HCr-labeled infected or mock-infected MOLT-3A cells as targets. The high titer human antisera and the fractionated antisera have been previously described. Before incubation with neutrophils, target cells were preincubated with specific antisera or normal human sera for 30 minutes at 37°C. ADCC was observed over a wide range of antibody concentrations; however, for specific antisera a concentration of 1:100 was optimal. A concentration of 1:25 was optimal for fractionated antisera. After a 4-hour incubation of neutrophils and target cells, supernatant fluids were removed to determine the extent of chromium release.

In neutrophil ADCC assays to the HTLV-II-infected B-lymphoblastoid cell line (J-WIL-2D), neutrophils were isolated from normal donors and CGD patients as described. The protocol for the ADCC assay was the same with this alternative target cell population except J-WIL-2D cells were preincubated with a 1:500 dilution of monoclonal antibody W632B (Pel Freeze [Rogers, AR]; specific for HLA-A,B,C monomorphic determinants) for 45 minutes at 22°C.

Percent lysis = (A – B)/C x 100%; where A is the mean counts per minute in the supernatant from wells containing target and neutrophils, B is the mean counts per minute from wells containing targets alone (representing spontaneous release of 3HCr), and C is the total counts per minute added to each well. Each measurement was done in triplicate. Spontaneous release of radio chromium was 14.2% ± 6.8% of the total.

RESULTS

We first examined neutrophils from healthy donors for cytotoxicity toward HIV-infected MOLT-3A cells. As shown in Fig 1, antibody-dependent neutrophil cytotoxicity to HIV-infected target cells was markedly enhanced by GM-CSF and G-CSF as a function of antibody concentration. Quantitatively similar results were obtained whether the neutrophils were obtained from normal donors (Fig 1A) or from AIDS patients (Fig 1B). We compared the levels of enhanced cytotoxicity at all concentrations of specific antisera and found no significant difference between the cytotoxic capability of neutrophils isolated from normal donors and the cytotoxic capability of neutrophils isolated from AIDS patients (P value = .7). The cytotoxic response of the neutrophils was dependent on the concentration of antisera used for preincubation with the HIV-infected target cells; maximal efficacy was seen at antibody dilutions of 1:100. The antibodies used in these studies were high titer antisera from patients with AIDS; thus, the neutrophil is directed toward the target by naturally occurring antibody to HIV. Levels of enhancement for neutrophil cytotoxicity are GM-CSF or G-CSF dose-dependent with maximal enhancement seen at concentrations of 100 pmol/L GM-CSF or 500 pmol/L G-CSF. These data indicate that both GM-CSF and G-CSF can enhance the neutrophils' inherent ability to kill HIV-infected lymphocytes irrespective of whether the neutrophils are from HIV-infected patients or healthy volunteers.

To determine the specificity of the antibody that mediated neutrophil cytotoxicity, we fractionated the immunoglobulins in one antiserum by affinity chromatography and obtained antibodies specific to particular HIV proteins. The specificities of the fractionated antisera are diagrammed in Fig 2 and have been confirmed by radioimmunoprecipitation of labeled HIV viral proteins. Antibodies to gpl20, the external envelope protein, were the most effective mediators of neutrophil cytotoxicity, whereas antibodies to p24 gag protein did not mediate an effective ADCC response against HIV-infected target cells (Fig 2). Levels of maximal ADCC enhancement toward HIV-infected targets were threefold to fivefold above basal levels of chromium release seen when
neutrophils were incubated with diluent control and target cells were incubated with specific antibody. When target cells were preincubated with normal human sera or fractionated antisera for the gag p24 of HIV, there was little neutrophil-mediated ADCC. Levels of neutrophil cytotoxicity were comparable whether neutrophils were isolated from normal donors or from AIDS patients. In the presence of fractionated antisera specific for env protein, GM-CSF-stimulated neutrophils from AIDS patients killed HIV-infected MOLT-3A cells in a manner equivalent to neutrophils obtained from normal donors. ADCC in the presence of anti-env antisera was specific for HIV-infected target cells because uninfected MOLT-3A target cells were not sensitive to neutrophil-mediated cytotoxicity with this antisera (Table 1).

Table 1. ADCC Toward Uninfected MOLT-3A (%) (fourfold) than that seen using GM-CSF–treated CGD neutrophils (sixfold).

DISCUSSION

We report here that both GM-CSF and G-CSF enhance AIDS patients' neutrophil cytotoxicity toward HIV-infected T lymphocytes in vitro. Without the addition of CSFs, levels of neutrophil cytotoxicity toward HIV-infected targets were significantly diminished. Neutrophil ADCC toward HIV-infected target cells is antibody-dependent and is most effectively mediated by antibodies recognizing the env protein of HIV. Our finding that antisera from AIDS patients is in agreement with other recently published reports; however, there is controversy in the literature whether antibodies with p24 specificity or antibodies with gp120 specificity effectively mediate ADCC toward HIV-infected targets. Our studies show that antibodies to gp120 are associated with maximal levels of neutrophil cytotoxicity. This result is consistent with studies by Ojo-Amaize et al using NK/K cell effector populations, which, like neutrophils, express Fc receptors for immunoglobulin and can actively mediate antibody-dependent cytotoxicity.

Although ADCC depends on effector cells with Fc receptors that facilitate binding to antibody-coated target cells, the mechanism of killing is not presently understood. In establishing a specific role for ADCC in immunity against

Table 2. Neutrophil ADCC (%)
AIDS, we are analyzing the specific components necessary for an effective cytotoxic response to virally infected target cells, and how to augment that response. In this regard, we found that neutrophils from CGD patients are capable of enhanced neutrophil ADCC toward HTLV-II and HIV-infected lymphocytes. CGD is a disorder characterized by recurrent bacterial and fungal infections caused by an impaired ability of the patients' phagocytic cells to produce microbicidal oxygen metabolites. Our results indicate that this respiratory burst is not an essential component for enhanced neutrophil ADCC toward HIV-infected targets, suggesting extracellular lytic mechanisms as proposed by Simone and Henkart.

A specific role for ADCC in host defense has not been defined in human retroviral infections. Recent studies in animal models with nonhuman retroviruses indicate host defense cells may be capable of destroying virus-infected cells as well as tumor cells in vivo. In AIDS, defense mechanisms, such as ADCC, may prove effective in limiting viral spread by destroying virus-infected cells in vivo. Our data support the idea that host defense cells, such as neutrophils, are capable of killing HIV-infected lymphocytes in vivo. Effector cells other than neutrophils have been shown to be active in mediating ADCC toward HIV-infected targets in vitro. Whether neutrophil ADCC or ADCC in general can play an active role in host defense against HIV remains to be determined; however, our data would suggest that GM-CSF and G-CSF can have a role in increasing neutrophil number and ADCC function in AIDS patients.

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