Monoclonal LYM-1 antibody-dependent cytolysis by human neutrophils exposed to GM-CSF: auto-regulation of target cell attack by cathepsin G

Luciano Ottonello,*[,1] Alan L. Epstein,[†] Marina Mancini,* Patrizia Dapino,* and Franco Dallegri*

Department of Internal Medicine, University of Genova Medical School, Italy; and [†]Department of Pathology, University of Southern California, Los Angeles

Abstract: Murine monoclonal antibody (mAb) Lym-1 is an immunoglobulin G2a specific for certain human leukocyte antigen-DR variants expressed on the surface of malignant B cells. It has been proposed for serotherapy in patients with B lymphomas. We have previously shown that mAb Lym-1 synergizes with granulocyte macrophage-colony stimulating factor to promote Raji B-lymphoid cell lysis by human neutrophils via the intervention of neutrophil Fc receptors type II and D-mannose-inhibitable interactions between CD11b–CD18 integrins and CD66b glycoproteins. Here, we provide evidence that the process is oxygen-independent by inference related to the release of primary granules and is regulated by cathepsin G activity. The lysis was indeed reproduced by replacing normal neutrophils with cells from three patients suffering from chronic granulomatous disease, i.e., neutrophils genetically incapable of generating oxidants. Moreover, the lysis was inhibited by the serine protease inhibitor 3,4-dichloroisocoumarin and by Z-glycyl-leucyl-phenyl-chloromethyl ketone (Z-Gly-Leu-Phe-CMK), which blocks cathepsin G. Conversely, the lysis was unaffected by N-methoxysuccinyl-alanyl-alanyl-prolyl-alanyl-CMK (MeOSuc-Ala-Ala-Pro-Ala-CMK; elastase inhibitor) and MeOSuc-Ala-Ala-Pro-valine (Val)-CMK, which inhibits elastase and proteinase 3. The ability of neutrophils, engaged in cytolysis, to release cathepsin G was proved by detecting this enzymatic activity spectrophotometrically and immunocytochemically. Moreover, inhibition of cathepsin G activity by concentrations of Z-Gly-Leu-Phe-CMK, incapable of affecting elastase activity, was found to reduce the release of elastase and myeloperoxidase from neutrophils under conditions similar to those used for cytolytic assays. These findings suggest that neutrophils auto-regulate their lytic efficiency by controlling the exocytosis of primary granules via their cathepsin G activity. *J. Leukoc. Biol.* 75: 99–105; 2004.

Key Words: *ADCC · lymphoma · primary granules · exocytosis · Fc receptors*

INTRODUCTION

In recent years, the antilymphoma monoclonal antibody (mAb) Lym-1 has been shown to trigger cellular cytotoxity through processes susceptible to amplification by cytokines [1, 2]. In this regard, mononuclear leukocytes and neutrophils have been shown to be active [2–4]. Moreover, neutrophils have been proved to be potent effector cells, particularly when stimulated by granulocyte macrophage-colony stimulating factor (GM-CSF) [3, 5]. These findings, coupled with the characteristics of the Lym-1 mAb, have suggested rational possibilities to raise new approaches to the treatment of lymphoproliferative disorders [6, 7]. In fact, Lym-1 mAb is a murine immunoglobulin G (IgG)2a antibody that recognizes a polymorphic variant of human leukocyte antigen-DR antigens present on the surface of B lymphoma cells [8]. Furthermore, these antigens are incapable of shedding or undergoing modulation after antibody binding [8]. Finally, the ability of Lym-1 to react with normal tissue cells including B lymphocytes is very low or absent [8]. In front of these attractive possibilities, a relatively modest knowledge is available about the molecular events underlying such a neutrophil-mediated activity. Recently, we found that neutrophils stimulated by Lym-1 mAb and GM-CSF exert cytolysis by using Fc receptors (FcR) type II as well as CD11b–CD18 integrins interacting with CD66b surface glycoproteins [9]. Nevertheless, the events occurring down-stream of the intervention of these membrane molecules are unknown. The present study provides evidence that the cytolytic process is oxygen-independent and is auto-regulated by a cathepsin G feedback activity modulating the exocytosis of primary granules.

MATERIALS AND METHODS

Culture medium and reagents

The following culture medium was used: RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated (56°C for 45 min) fetal calf serum (FCS; Hyclone Europe Ltd., Cramlington, NE) and 2 mM glutamine

[1] Correspondence: Dipartimento di Medicina Interna e Specialità Mediche, Viale Benedetto XV n.6, I-16132 Genova, Italy. E-mail: otto@unige.it

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(Irvine Scientific). Hanks' balanced salt solution (HBSS) was from Irvine Scientific. Ficoll-Hypaque was purchased from Seromed (Berlin, Germany). Sodium chromate ^{51}Cr was from Radiochemical Centre (Amersham, UK). Triton X-100, zymosan A, phorbol 12-myristate 13-acetate (PMA), human albumin, rabbit anti-human albumin IgG, ethidium bromide, fluorescein diacetate, catalase, superoxide dismutase (SOD), methionine, taurine, mannitol, benzoate, D-mannose, galactose, 3,4-dichloroisocoumarin, succinyl-alanyl-alanyl-phenyl-p-nitroanilide, N-methoxysuccinyl-alanyl-alanyl-prolyl-valine-p-nitroanilide, and N-methoxysuccinyl-alanyl-alanyl-prolyl-valine-chloromethyl ketone (MeOSuc-Ala-Ala-Pro-Val-CMK) were purchased from Sigma Chemical Co. (St. Louis, MO). Z-glycyl-leucyl-phenyl-CMK (Z-Gly-Leu-Phe-CMK) was from Enzyme Systems Products (Livermore, CA). MeOSuc-Ala-Ala-Pro-Ala-CMK was from Bachem AG (Bubendorf, Switzerland). 3,3′,5,5′-Tetramethyl benzidine was from ICN Biochemicals (Aurora, OH). Heparin was obtained from Roche (Milano, Italy). Human recombinant GM-CSF was from Genzyme (Cambridge, MA). Giemsa was purchased from Merck (Darmstadt, Germany). Lym-1, the mAb described previously [8], was used as an antitarget mAb for the cytolytic assay. Mouse IgG2a isotype-control mAb CLB 601 was from RDI (Flanders, NJ). Anti-CD32 IV.3 Fab fragments and anti-CD16 3G8 $F(ab')_2$ fragments were from Medarex (West Lebanon, NH). Rabbit anti-human cathepsin G was from Calbiochem (San Diego, CA).

Neutrophil preparation

Heparinized venous blood (10 U/ml heparin) was obtained from healthy male volunteers after informed consent. Moreover, neutrophils were obtained form three patients described previously with chronic granulomatous disease (CGD) [10], congenitally incapable of generating nicotinamide adenine dinucleotide phosphate oxidase-dependent oxidants, as tested by SOD-inhibitable cytochrome c reduction, as described previously [11]. Neutrophils were isolated by dextran sedimentation and subsequent centrifugation on a Ficoll-Hypaque density gradient, as described previously [9]. Contaminating erythrocytes were removed by hypotonic lysis [9]. Then, neutrophils were washed three times with HBSS and resuspended in incubation medium at appropriate concentrations. For experiments of enzyme release, incubation medium was replaced with HBSS. Final cell suspension was greater than 97% pure and greater than 98% viable, as determined by standard assays [9].

Cytolytic assay

Cytolytic activity of purified neutrophils was studied, as described previously in detail [9]. Briefly, Raji cells were grown in RPMI-FCS medium and used as targets. For the assay, 5×10^6 Raji cells were labeled with 100–200 μCi sodium chromate ^{51}Cr by incubating for 1 h at 37°C. After washing, labeled cells were resuspended in RPMI-FCS. Then, target cells (2×10^4) were mixed with neutrophils at an effector:target (E:T) ratio of 20:1 in the presence of 10 μg/ml mAb Lym-1 and 1 ng/ml GM-CSF, as described previously [9]. These experimental conditions were chosen on the basis of several tests with different E:T ratios and GM-CSF and Lym-1 dose-response curves, as previously reported [3]. The chosen concentrations of GM-CSF and Lym-1 are those inducing the maximal activity [3]. Furthermore, the Lym-1 concentration used in the present setting is nearly threefold higher than the dissociation constant for its antigen in the Raji cell surface [8]. Tests were performed in triplicate. After 14 h of incubation in a humidified atmosphere of 95% air and 5% CO_2, the ^{51}Cr release was determined in the cell-free supernatants. The percentage of cytolysis was calculated according to the equation, $100 \times (E–S)/(T–S)$, in which E is the counts per minute (cpm) released in the presence of effector cells, T is the cpm released after lysing targets with 5% Triton X-100, and S is the cpm spontaneously released by target cells incubated with medium alone [9]. Control experiments were always performed to assess the effect of each reagent on effector and target cell viability (always <5%), as determined by standard assays [9].

Release of cathepsin G

The release of cathepsin G was performed under conditions similar to those used for cytolytic assays. Briefly, 4×10^5 neutrophils were mixed with 2×10^4 Raji cells in the presence of Lym-1 mAb, with and without GM-CSF (final volume: 100 μl). Tests were performed in triplicate, using a round-bottom microplate (Falcon). Experiments were performed in RPMI 1640 supplemented with 0.1 mg/ml human albumin. The substrate of cathepsin G (Suc-Ala-Ala-Phe-p-nitroanilide) was mixed with the cells at the beginning of the assay, at the concentration of 0.5 mM. After 4 h of incubation, microplates were centrifuged, and 50 μl of the cell-free supernatants was read at 405 nm in a microplate reader (Titertek TwinReader Plus, Huntsville, AL). Experiments were also performed by testing cathepsin G activity in supernatants from cells incubated in the absence of the enzyme substrate.

Release of elastase

The release of elastase was measured under conditions similar to those used for cytolytic assays. Briefly, 4×10^5 neutrophils were mixed with 2×10^4 Raji cells in the presence of Lym-1 mAb, with and without GM-CSF (final volume: 100 μl). Moreover, neutrophils were also stimulated with PMA or albumin-antialbumin-immune complexes (prepared by incubating human albumin and rabbit anti-human albumin IgG at equivalence, which was determined on the basis of quantitative precipitin curves at the molar ratio 1:5). Tests were performed in triplicate, using a round-bottom microplate (Falcon). Experiments were performed in RPMI 1640 supplemented with 0.1 mg/ml human albumin. The substrate of elastase (MeOSuc-Ala-Ala-Pro-Val-p-nitroanilide) was mixed with the cells at the beginning of the assay, at the concentration of 0.5 mM. After 4 h of incubation, microplates were centrifuged, and 50 μl of the cell-free supernatants was read at 405 nm in a microplate reader (Titertek TwinReader Plus, Huntsville, AL).

Release of myeloperoxidase

The release of myeloperoxidase was measured under conditions similar to those used for cytolytic assays. Briefly, 4×10^5 neutrophils were mixed with 2×10^4 Raji cells in the presence of Lym-1 mAb, with and without GM-CSF (final volume: 100 μl). Moreover, neutrophils were stimulated with PMA or albumin-antialbumin-immune complexes (prepared by incubating human albumin and rabbit anti-human albumin IgG at equivalence, which was determined on the basis of quantitative precipitin curves at the molar ratio 1:5). Tests were performed in triplicate, using a round-bottom microplate (Falcon). Experiments were performed in RPMI 1640 supplemented with 0.1 mg/ml human albumin. The substrate of myeloperoxidase 3,3′,5,5′-tetramethylbenzidine was mixed with the cells at the beginning of the assay, at the concentration of 2.8 mM. After 4 h of incubation, microplates were centrifuged, and 50 μl of the cell-free supernatants was read at 414 nm in a microplate reader (Titertek TwinReader Plus, Huntsville, AL).

Immunocytochemical neutrophil staining

Neutrophils were incubated with Raji target cells at an E:T ratio of 5:1 (2×10^4 Raji cells) in the presence of Lym-1 mAb, with and without GM-CSF (final volume: 100 μl) as for cytolytic assays. After incubation for 15 min at 37°C, samples were centrifuged to favor cell interactions, and then the cells were resuspended. Immunocytochemical cell staining was performed on cytopreps, using a rabbit anticathepsin G antibody (IgG) and a biotinylated second antibody as a linker between the primary antibody and the streptavidin-peroxidase conjugate. The reaction was performed according to the instructions of the manufacturer (Zymed Laboratories Inc., South San Francisco, CA).

Statistical analysis

Data are expressed as mean ± 1 SD. Statistical differences were determined by the Kruskal-Wallis test followed by Dunn's multiple comparison test. Significance was accepted when $P < 0.05$.

RESULTS

Neutrophil-mediated Lym-1 antibody-dependent cellular cytotoxicity (ADCC) as an oxygen-independent process

Consistent with our previous observations [9], human neutrophils failed to lyse ^{51}Cr-labeled Raji target cells (**Fig. 1**). Moreover, the addition of the antitarget mAb Lym-1 to the system resulted in low but significant levels of lysis (Fig. 1).

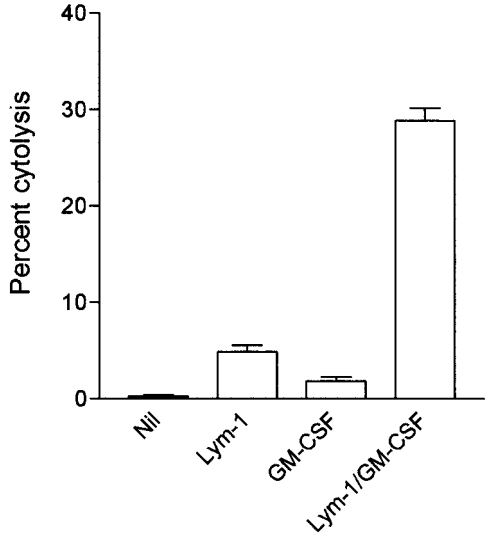

Fig. 1. Lym-1 mAb-dependent lysis of Raji cells by human neutrophils in the presence of GM-CSF. ^{51}Cr-labeled Raji cells were at 2×10^4/well. The neutrophil:target cell ratio was 20:1. Lym-1 = 10 μg/ml; GM-CSF = 1 ng/ml; incubation time = 14 h. Results are expressed as mean ± 1 SD. Nil: n = 21; Lym-1: n = 113; GM-CSF: n = 35; Lym-1 plus GM-CSF: n = 113; Lym-1 versus Nil: $P < 0.01$; Lym-1 plus GM-CSF versus Nil 1: $P < 0.001$.

Conversely, GM-CSF, in the absence of Lym-1, had no effect (Fig. 1). Finally, the simultaneous addition of Lym-1 and GM-CSF to neutrophil-target cell cocultures resulted in consistent amplification of the lysis (Fig. 1); i.e., GM-CSF synergistically cooperates with the antitarget mAb to stimulate neutrophil cytolytic activity. In accord with earlier observations [3], neutrophils were incapable of inducing Raji cell lysis when Lym-1 was replaced by an isotype-matched control mAb (data not shown). Lym-1-mediated neutrophil cytolysis was unaffected by adding scavengers of oxidants such as SOD (superoxide anion scavenger), catalase (which degrades hydrogen peroxide), taurine, methionine (hypochlorous acid scavengers), mannitol, and benzoate (hydroxyl radical scavengers; **Fig. 2A**). Moreover, to test the actual intervention of oxygen-independent versus oxygen-dependent cytolytic systems, neutrophils from three CGD patients were tested as effectors. Neutrophils from CGD are indeed incapable of generating oxidants. As these cells were found to be efficient effectors of GM-CSF-stimulated Lym-1 ADCC (Fig. 2B), the process appears to involve oxygen-independent lytic pathways.

Intervention of proteases in GM-CSF-stimulated Lym-1 ADCC by neutrophils

As shown in **Figure 3**, Lym-1 mAb-dependent lysis by GM-CSF-stimulated neutrophils was efficiently inhibited in a dose-dependent manner by 3,4-dichloroisocoumarin, a known inhibitor of serine proteases [12]. Dose-dependent suppression of the neutrophil activity could be also observed using Z-Gly-Leu-Phe-CMK (Fig. 3), which blocks cathepsin G [13]. Conversely, lysis was unaffected by MeOSuc-Ala-Ala-Pro-Val-CMK (Fig. 3), which inhibits elastase and proteinase 3 [14] and by MeOSuc-Ala-Ala-Pro-Ala-CMK, a specific inhibitor of neutrophil elastase [15], also at concentrations 100-fold higher than Z-Gly-Leu-Phe-CMK and 3,4-dichloroisocoumarin (Fig. 3). 3,4-Dichloroisocoumarin and Z-Gly-Leu-Phe-CMK, at the concentrations used in the Lym-1 mAb cytolytic system, were found to act as efficient inhibitors of cathepsin G activity in supernatants from neutrophils exposed to opsonized zymosan particles (data not shown). These data are consistent with the intervention of cathepsin G in the neutrophil-mediated cytolytic process.

Release of cathepsin G by neutrophils engaged in Lym-1 ADCC

Studies were performed to prove that the release of cathepsin G by neutrophils is involved in Lym-1 ADCC. Supernatants from neutrophils, coincubated with Raji cells up to 4 h in the presence of GM-CSF and Lym-1 mAb, did not contain detectable amounts of cathepsin G activity. On the contrary, when the cell incubation was performed in the presence of the substrate of cathepsin G, the addition of Lym-1 mAb to the coculture of neutrophils and Raji cells resulted in the detection of measurable amounts of cathepsin G activity. In fact, neutrophils (4×10^5) incubated with Raji cells (2×10^4) were found to release only 0.69 ± 0.96% of their cathepsin G (mean±1 SD; n=12), whereas in the presence of Lym-1 mAb, they released 4.94 ± 1.65% of their enzyme content (mean±1 SD; n=15). The release of the enzymatic activity was further enhanced by the addition of GM-CSF, per se, inactive when used in the absence of Lym-1 mAb. In the presence of GM-CSF and Lym-1 mAb, the percentage of the enzyme released by neutrophils was 8.03 ± 2.87 (mean±1 SD; n=15). These data suggest that cathepsin G, released by neutrophils engaged in the cytolytic function, remains associated with the cells. Consistent with this

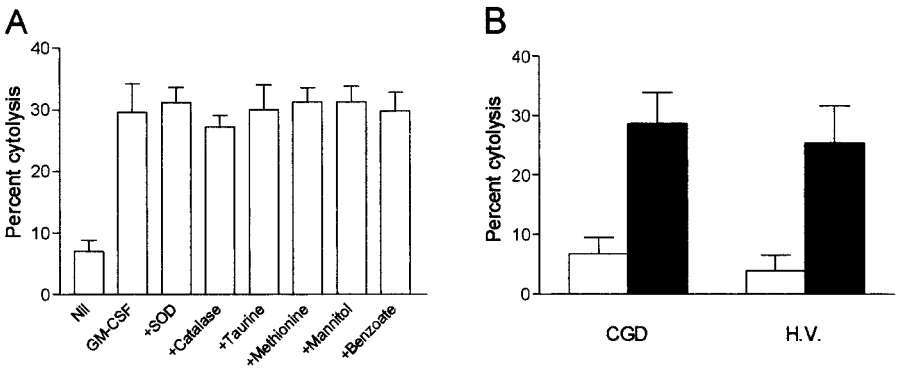

Fig. 2. Role of oxidants in Lym-1 mAb-dependent lysis of Raji cells. (A) Effect of oxidant scavengers and inhibitors on Lym-1 mAb-dependent lysis of Raji cells. SOD: 300 U/ml; catalase: 4000 U/ml; taurine: 20 mM; methionine: 20 mM; mannitol: 20 mM; benzoate: 20 mM. ^{51}Cr-labeled Raji cells were at 2×10^4/well. The neutrophil:target cell ratio was 20:1. Lym-1 = 10 μg/ml; GM-CSF = 1 ng/ml; incubation time = 14 h. (B) Lym-1 mAb-dependent lysis of Raji cells by neutrophils from three patients with CGD. H.V., Healthy volunteers. Open bars: Absence of GM-CSF; solid bars: presence of GM-CSF. ^{51}Cr-labeled Raji cells were at 2×10^4/well. The neutrophil:target cell ratio was 20:1. Lym-1 = 10 μg/ml; GM-CSF = 1 ng/ml; incubation time = 14 h.

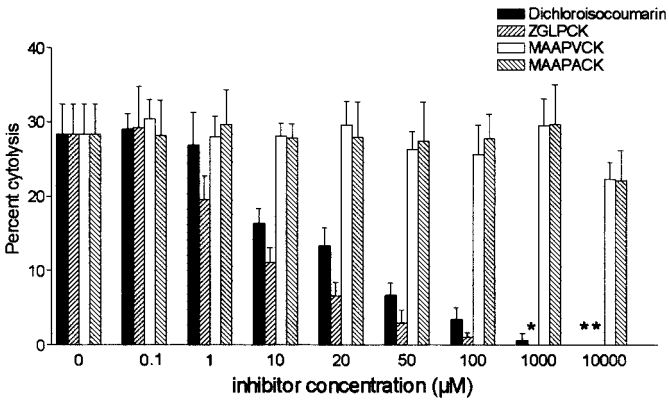

Fig. 3. Effect of 3,4-dichloroisocoumarin, Z-Gly-Leu-Phe-CMK, MeOSuc-Ala-Ala-Pro-Val-CMK, and MeOSuc-Ala-Ala-Pro-Ala-CMK on the GM-CSF-stimulated, neutrophil-mediated, Lym-1-dependent lysis. 3,4-Dichloroisocoumarin (serine proteases inhibitor); Z-Gly-Leu-Phe-CMK (ZGLPCK; cathepsin G inhibitor); MeOSuc-Ala-Ala-Pro-Val-CMK (MAAPVCK; elastase and proteinase 3 inhibitor); MeOSuc-Ala-Ala-Pro-Ala-CMK (MAAPACK; elastase inhibitor). ^{51}Cr- labeled Raji cells were at 2×10^4/well. The neutrophil:target cell ratio was 20:1. Lym-1 = 10 μg/ml; GM-CSF = 1 ng/ml; incubation time = 14 h. Number of experiments = four. *, Not performed.

conclusion, the enzyme could be detected at sites of neutrophil-target cell interaction (**Fig. 4**).

Effects of inhibitors of Lym-1 ADCC on the release of cathepsin G by neutrophils

Previous observations suggest that neutrophil-mediated Lym-1 ADCC absolutely requires FcRII (inhibitable by specific mAb IV.3) as well as the lectin-like (i.e., mannose-inhibitable) interaction between neutrophil carcinoembryonic antigen (CEA)-related glycoproteins (CD66b) and CD18 integrins [9]. Consequently, these inhibitors of neutrophil-mediated ADCC (mAb IV.3 and D-mannose) were tested for their effects on the neutrophil ability to exhibit the release of cathepsin G during cytolysis. As shown in **Figure 5**, mAb IV.3 and D-mannose, at concentrations able to inhibit neutrophil cytolysis [6], significantly reduced the release of cathepsin G by neutrophils engaged in ADCC. Conversely, mAb 3G8, specific for FcγRIII and control sugar galactose, was completely inactive (Fig. 5).

Regulatory role of cathepsin G in neutrophil-mediated cytolysis

As cathepsin G is located in primary granules of neutrophils, experiments were undertaken to demonstrate if the observed requirements for this enzyme activity in the cytolytic process reflect the actual intervention of primary granules in the lytic process. In fact, neutrophils engaged in ADCC were found to release other components of primary granules, i.e., elastase and myeloperoxidase. Moreover, the addition of the cathepsin G inhibitor to a mixture of neutrophils, Raji cells, Lym-1 mAb, and GM-CSF resulted in the inhibition of the release of elastase and myeloperoxidase from neutrophils (**Fig. 6**). As shown in Figure 6, this inhibitor was also found to inhibit efficiently the release of the enzymes from neutrophils incubated with albumin-antialbumin-immune complexes. On the contrary, the inhibitor was ineffective using neutrophils stimulated with PMA (Fig. 6).

DISCUSSION

The present data show that the lysis of B-lymphoma Raji targets by normal neutrophils, synergistically stimulated by the antitarget mAb Lym-1 and GM-CSF, is oxygen-independent. This is suggested by different findings. First, the lysis could be detected by replacing normal neutrophils with cells from three patients with CGD, i.e., cells incapable of generating oxidants [10, 11]. Second, the lysis was unaffected by a variety of

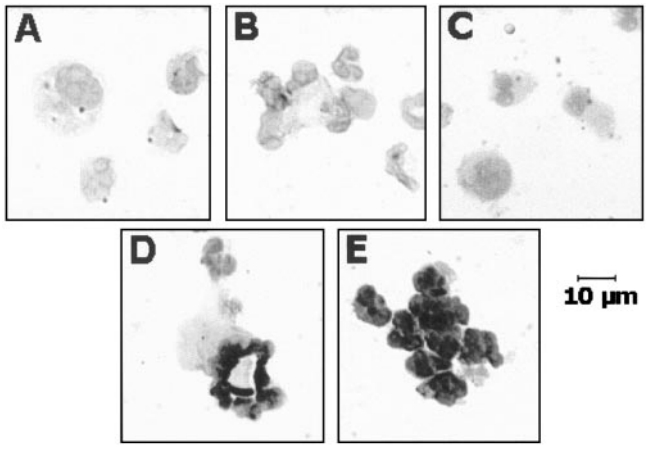

Fig. 4. Cell-surface expression of cathepsin G at sites of E:T interaction. Neutrophils and Raji cells were incubated at a 5:1 ratio, under conditions similar to those used for cytolitic assays. Lym-1 = 10 μg/ml; GM-CSF = 1 ng/ml; opsonized zymosan: 1 mg/ml. Immunocytochemical cell staining was performed as reported in Materials and Methods. (A) Neutrophils incubated with Raji cells. (B) Neutrophils incubated with Raji cells in the presence of Lym-1. (C) Neutrophils incubated with Raji cells in the presence of GM-CSF. (D) Neutrophils incubated with Raji cells in the presence of Lym-1 and GM-CSF. (E) Neutrophils incubated with opsonized zymosan.

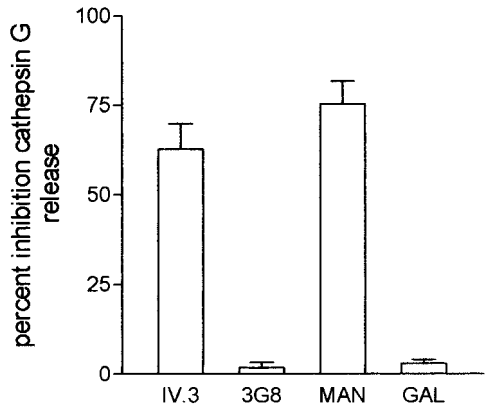

Fig. 5. Effect of anti-FcRII mAb IV.3, anti-FcRIII mAb 3G8, D-mannose, and galactose on the neutrophil cathepsin G release. The release was studied by incubating neutrophils with Raji cells for the cytolytic assays. Labeled Raji cells were at 2×10^4/well. The neutrophil:target cell ratio was 20:1. Lym-1 = 10 μg/ml; GM-CSF = 1 ng/ml; mAb IV.3 and mAb 3G8 = 4 μg/ml; D-mannose (MAN) and galactose (GAL) = 100 mM. The results are expressed as mean ± 1 SD of six (IV.3 and 3G8) and 11 (MAN and GAL) experiments. mAb IV.3 versus control: $P < 0.01$. D-mannose versus control: $P < 0.001$.

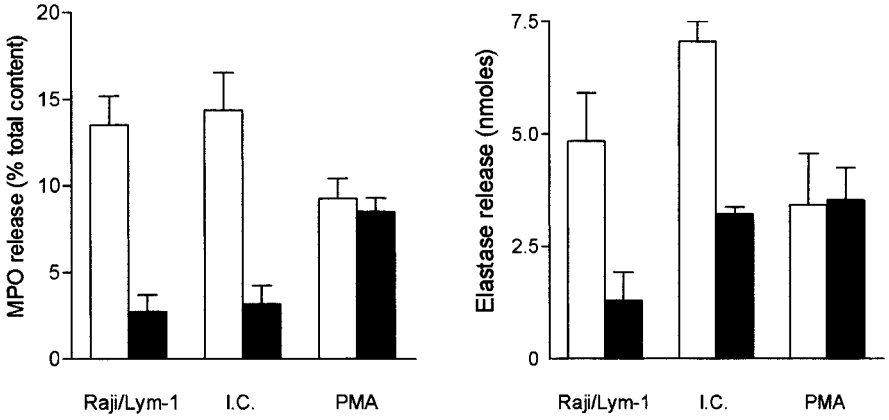

Fig. 6. Effect of the cathepsin G inhibitor Z-Gly-Leu-Phe-CMK on neutrophil elastase and myeloperoxidase release. Neutrophils were stimulated by Lym-1 mixed with GM-CSF and Raji cells as for cytolysis (Raji/Lym-1) with albumin-antialbumin-immune complexes (I.C.) or with PMA. Z-Gly-Leu-Phe-CMK = 100 μM. Open bars: Enzyme release in the absence of Z-Gly-Leu-Phe-CMK; solid bars: enzyme release in the presence of Z-Gly-Leu-Phe-CMK. (A) Myeloperoxidase (MPO) release: Results are expressed as percent of total content (mean±1 SD of six experiments). Raj/Lym-1: $P < 0.01$; I.C.: $P < 0.01$; PMA: $P > 0.05$. (B) Elastase release: Results are expressed as nmoles enzyme activity released (mean±1 SD of six experiments for Raji/Lym-1 and I.C. or five experiments for PMA). Raj/Lym-1: $P < 0.05$; I.C.: $P < 0.05$; PMA: $P > 0.05$.

scavengers of different neutrophil-derived oxidants, used at concentrations known to be effective in oxygen-dependent cytolytic systems [16]. Moreover, this conclusion is consistent with our previous observations showing the intervention of oxygen-independent mechanisms in the lysis of Raji cells by neutrophils in the presence of heterologous antitarget antiserum [17]. In addition, evidence for the ability of neutrophils to exert nonoxidative lysis has been reported in other ADCC systems, using Herpes simplex virus-infected human embryonic lung cells as targets [18] and in a way more closely related to the present results, mAb 3F8-sensitized human melanoma and neuroblastoma cells [19]. Finally, our data are in agreement with the ability of GM-CSF to prime and/or stimulate nonoxidative effector cell responses in neutrophils [20], including ADCC toward human immunodeficiency virus-infected targets [21]. At present, defensins, stored in primary granules [22], which are able to lyse various tumor cells including Raji cells [23], represent the major candidates for the cytolysin role. Consistent with this possibility, neutrophil ADCC has been proven to be a process dependent on the release of primary granules [24].

Various cell-surface structures, such as certain FcRs for IgG (FcγR), β_2 integrins, and other molecules, have been shown to intervene in the neutrophil-mediated lysis of mAb-sensitized tumor targets [9, 25]. Furthermore, and in agreement with findings reported for other cytotoxic cells [26], neutrophils are known to exert ADCC toward targets sensitized by heterologous antibodies via the intervention of serine esterases or proteases [27]. In this regard, two recent reports have highlighted the essential role of β_2 integrins for E:T adhesion, azurophil granule exocytosis and ADCC [28, 29]. The present results not only confirm these findings but also contribute to the understanding of the subsequent events underlying neutrophil cytolytic triggering. Indeed, under the present conditions, the lytic event was found to be suppressed by dichloroisocoumarin, a general serine protease inhibitor already shown to be effective in certain models of lymphocyte cytotoxicity [30]. Moreover, a low molecular weight compound (Z-Gly-Leu-Phe-CMK), used at a concentration able to inhibit cathepsin G without affecting elastase activity, displayed potent inhibitory effects in the neutrophil Lym-1/ADCC system. Consistently, cathepsin G activity was detected by adding the enzyme substrate directly to the ADCC system. Furthemore, using an immunocytochemical staining assay, cathepsin G was detected at sites of E:T cell contact. Therefore, the present findings are consistent with the evidence that activated neutrophils can express on their plasma membrane constituents of primary granules including cathepsin G [31]. In this regard, it has been shown that activated neutrophils have $\sim 4 \times 10^6$ cathepsin G-binding sites per cell [31]. As for the cytolytic process, Lym-1 mAb and GM-CSF were found to synergistically stimulate the release of the cathepsin G. Moreover, the release of the enzyme activity by neutrophils is down-regulated by exogenous reagents, such as anti-FcRII mAb IV.3 and D-mannose, in a way parallel to the effects displayed by these reagents in the cytolytic system. Taken together, the data suggest that the neutrophil-mediated Lym-1 ADCC and the neutrophil surface expression of active cathepsin G are tightly associated events.

It has been found previously that cathepsin G binds to human lymphocytes, especially natural killer (NK) cells [32]. Moreover, it has been reported that this enzyme acts as a potent stimulator of NK cytotoxicity [33]. The binding of proteolytically active cathepsin G to NK cells is indeed sufficient to trigger signals necessary for the cell activation with the consequent granule exocytosis [33]. In the present neutrophil ADCC system, cathepsin G activity appears to be crucial to permit an efficient exocytosis of primary granules, as suggested by the ability of the cathepsin G inhibitor Z-Gly-Leu-Phe-CMK to reduce the release of elastase or myeloperoxidase used as alternative markers of primary granules. The ability of cathepsin G to regulate the exocytosis of primary granules during neutrophil-mediated Lym-1 ADCC is an event that seems to be reproduced only under experimental conditions involving FcR activation. In fact, as shown above, cathepsin G activity was found to be involved in the triggering of primary granule exocytosis from neutrophils challenged with immune complexes but not with cells exposed to PMA. The natural enzymatic substrate or substrates of cathepsin G during neutrophil ADCC are unknown. As suggested for the above-mentioned NK activity [33], the enzyme might act by digesting surface molecules involved in the regulation of the cell exocytosis, in turn controlling the cell lytic efficiency.

Recently, it has been shown that neutrophils from cathepsin G-deficient mice display normal chemotaxis, oxidative metabolism, and phagocytosis [34]. As far as the microbicidal activity is concerned, two recent reports show an impaired resistance of cathepsin G-deficient mice to *Aspergillus fumigatus* and *Staphylococcus aureus* infections [35, 36], whereas other authors

observed normal survival of cathepsin G-deficient mice after Gram-negative and Gram-positive bacterial challenge [34]. Conversely, in vitro studies have indicated a new, putative role for neutrophil cathepsin G, taking into account some similarities with lymphocyte granzyme B [37]. Cathepsin G was indeed capable of inducing apoptosis of certain cells by activating caspase cascade [38, 39]. Thus, it has been suggested that in addition to defensins, cathepsin G could be a death mediator for neutrophil-dependent ADCC [40]. Furthermore, we provide evidence for another activity of cathepsin G, in this case relevant for the control of the actual ability of neutrophils to express their tumoricidal potential. In other words, neutrophils, by means of a cathepsin G feedback activity, auto-regulate their own capability to degranulate and exert ADCC. In general, this seems to agree with previous data showing that the lysosomal enzyme release by neutrophils is under the control of serine proteolytic activities [27] and also with the evidence that cathepsin G stimulates secretion from certain nonphagocytic cells such as gland-serous cells [41].

In summary, our results, together with data from previous reports [9, 28, 29], define neutrophil ADCC as a GM-CSF-sensitive multistep process, including FcRII, by which neutrophils recognize Ab-sensitized target cells (**Fig. 7A**); CD11b/CD18 integrins, associated with CD66b CEA-like molecules via lectin-like interactions, crucial for the spreading onto the targets (Fig. 7B); and membrane-bound cathepsin G molecules, which are strictly involved in triggering the delivery of a primary, granule-dependent lethal hit from neutrophil (Fig. 7C).

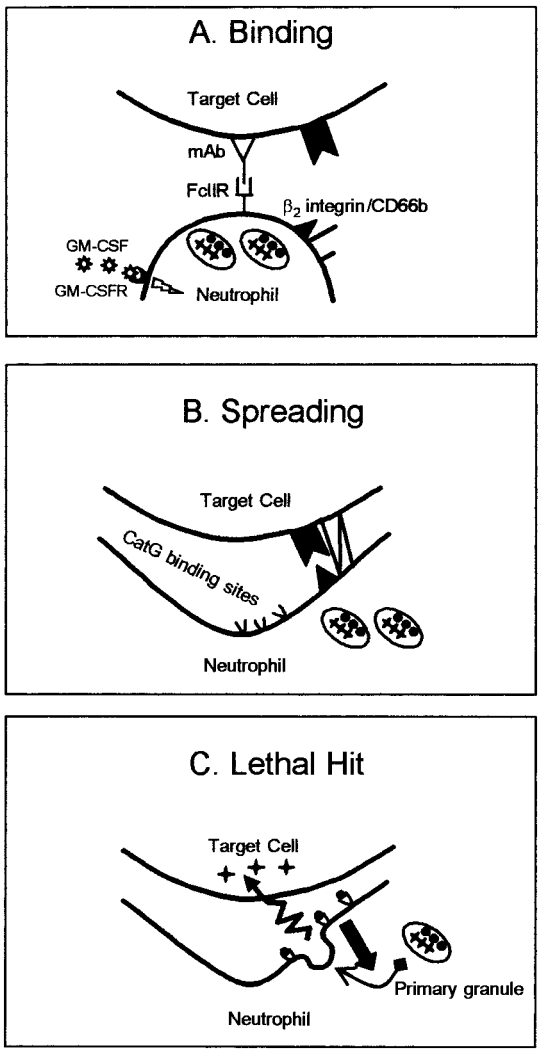

Fig. 7. Proposed model for the role of cathepsin G in neutrophil ADCC: cooperation with FcR, CD11b/CD18 β2 integrins, and CD66b–CEA-like molecules. (A) Neutrophil recognizes and binds to mAb-sensitized target cell via FcRII [9, 26, 27]. (B) Neutrophil spreads onto the target cell. Spreading depends on the activation of β2 integrins physically linked to CD66b via lectin-like interactions [9, 26, 27]. (C) At site of interaction with target cell, activated neutrophil exposes cathepsin G (CatG) molecules on the outer membrane surface. Membrane-bound cathepsin G amplifies primary granule exocytosis with consequent release of the lethal hit in proximity of the target cell (present results).

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