Mechanism of Target Cell Recognition by Natural Killer Cells: Characterization of a Novel Triggering Molecule Restricted to CD3⁻ Large Granular Lymphocytes

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Summary

In an attempt to identify a molecule in target recognition by CD3⁻ large granular lymphocytes (LGL), we have generated a rabbit antiidiotypic (anti-ID) serum against a monoclonal antibody (mAb 36) that reacted with the cell membrane of K562. Flow cytometry analysis demonstrated that the anti-ID serum bound selectively to CD3⁻ LGL and that $F(ab')_2$ fragments of the anti-ID serum blocked both target cell binding and lysis by NK cells. Stimulation of CD3⁻ LGL with $F(ab')_2$ fragments resulted in the release of serine esterases and the secretion of interferon γ. Furthermore, anti-ID $F(ab')_2$ antibodies crosslinked to anti-DNP $F(ab')_2$ mediated directed cytotoxicity of a non–natural killer (NK)-susceptible mouse target (YAC-1) via this surface ligand. These functional reactivities were only removed by adsorption with the specific idiotype. Protein analysis showed that the anti-ID serum immunoprecipitated 80-, 110-, and 150-kD proteins. Using this anti-ID, a partial cDNA was cloned and an antipeptide antiserum was made against the portion of the predicted amino acid sequence that corresponded to a portion of the ID binding region. This antipeptide serum exhibited similar functional and biochemical reactivities to those observed with the anti-ID serum. These data suggest that the cell surface moiety recognized by the anti-ID and anti-p104 is novel and is selectively involved in both recognition and triggering of NK-mediated lytic function.

NK activity has been hypothesized as the first line of defense against spontaneously developing tumor cells and many microorganisms (1–5). In mice, rats, and humans, this cytotoxic activity is mediated predominately by CD3⁻ LGL (6–9). This lymphocyte subset also demonstrates antibody-dependent cellular cytotoxicity (ADCC)[1] (2) and has the capacity to secrete lymphokines that augment or suppress cell-mediated immunity (5, 10, 11). In addition, LGL secrete IFN-γ in response to the interaction of CD3⁻ LGL with NK target cells, presumably through triggering of appropriate recognition receptors (6).

A model has recently been proposed that defines the mechanism of cytotoxicity by LGL in two clearly distinguishable steps (12). The first step is recognition and binding of the LGL to the target cells (6, 12–14), while the second step includes post-binding events that lead to lysis of the target cell. Studies have shown that target recognition by CD3⁻ LGL does not involve the TCR and that CD3⁻ LGL lack gene rearrangement and expression of the α, β, and γ chains of the TCR (6, 13–18). Furthermore, target recognition is not mediated via the Fc receptor that functions in LGL-mediated ADCC killing (12). Thus, the mechanisms of target cell recognition and triggering of lysis by LGL remain unclear.

In the present study, an antiidiotypic (anti-ID) antiserum directed against a mAb (mAb 36) (Bino, T., J. Rossio, J. L.

[1] *Abbreviations used in this paper:* ADCC, antibody-dependent cellular cytotoxicity; anti-ID, antiidiotypic; BBS, borate-buffered saline; DTNB, dithiobis (2-nitrobenzoic acid); KLH, keyhole limpet hemocyanin; NRS, normal rat serum; SMC, succinimidyd trans-4-(*N*-maleimidyl-mathyl) cyclohexane-1-carboxylate.

Frey, and J. R. Ortaldo, manuscript in preparation) that blocked the binding and lysis of $CD3^-$ LGL to NK-susceptible target cells was generated in rabbits. This anti-ID antiserum was shown to react with human LGL and to specifically inhibit the LGL-mediated binding and lysis of tumor targets. Moveover, the anti-ID antiserum activated the lytic mechanism of LGL based on its ability to mediate redirected lysis of NK-resistant targets. Furthermore, pretreatment of LGL with the anti-ID antiserum perturbed the membrane of the LGL and triggered the release of serine esterases and IFN-γ. This anti-ID antiserum has been used to isolate a novel partial cDNA clone from a human NK cDNA λ-gt11 expression library (Anderson, S. K., S. Gallinger, R. Roder, J. L. Frey, H. Young, and J. R. Ortaldo, manuscript in preparation). Based on the predicted protein sequence of the cDNA, a synthetic peptide (p104) was made with an amino acid sequence that corresponded to a portion of the idiotype binding region of this gene. The anti-p104 serum reacted with LGL and demonstrated functional properties in a manner similar to that of the anti-ID antiserum. Although other cell surface structures may exist on $CD3^-$ LGL that also may be involved in the binding of NK cells to NK-susceptible targets, the surface moiety we have described appears to be a very important structure in the recognition and lysis of tumor targets by NK cells.

Materials and Methods

Cell Lines and Antibodies. K562, derived from a patient with chronic myelogenous leukemia in blast crisis (19); MOLT-4, a T lymphoid cell line derived from a patient with acute lymphocytic leukemia (20); and FeMX, a melanoma cell line (6), were cultured in RPMI 1640 (BioFluids, Inc., Rockville, MD) supplemented with 10% heat-inactivated FCS (Biofluids, Inc.), 100 μg/ml of gentamicin, and 0.6 ng/ml of L-glutamine (Biofluids, Inc.).

Source and Preparation of Human PBL. Leukocytes were separated from buffy coats of peripheral blood by centrifugation on Ficoll-Hypaque gradients. Human LGL and T cells were prepared as previously described (21, 22). Monocytes were isolated from plastic dishes and thoroughly washed with PBS, and collected by treatment of the plastic dishes with Versene solution (Bethesda Research Laboratories, Gaithersburg, MD) and scraping. LGL-enriched preparations contained 80–90% LGL as determined by morphological analysis of Giemsa-stained cytocentrifuge preparations, and were 70–75% $CD11^+$, 75–80% $CD16^+$, and <5% $CD3^+$ as determined by flow cytometry. Purified T cell preparations contained <1% LGL and were >95% $CD3^+$. The cells were cultured in RPMI 1640 supplemented with 10% human AB serum (M. A. Bioproducts, Walkersville, MD), 0.1 mM nonessential amino acid, 2 mM sodium pyruvate, 4 mM L-glutamine, 50 μg/ml gentamicin and 2.5×10^{-5} mM 2-ME (Bio-Rad Laboratories, Richmond, CA). Rat LGL and T cells were also obtained from peripheral blood and prepared as previously described (23).

Preparation of Rabbit Anti-ID Antibody. Three rabbits were initially immunized with a subcutaneous injection of an emulsion of 0.5 ml of 100 μg of affinity-purified mAb 36 (IgM) mixed with an equal volume of CFA. After 14 d, another subcutaneous injection of 100 μg mAb 36 mixed with an equal volume of IFA was administered. 7 d later, the rabbits were bled, and the serum was prepared. Antiisotype antibody was removed from the serum using a control mouse IgM, MOPC 104E (Litton Bionetics, Charleston, SC) Sepharose 4B IgM affinity column. Nonbound eluted material was partially purified by precipitation with 35% ammonium sulfate and an ELISA for anti-ID antibody was performed on microtiter plates that had been coated with mAb 36 or with MOPC 104E. Positive results on mAb 36 plates with parallel negative results on MOPC 104E confirmed the presence of the anti-ID antibody. Prebleed normal rabbit serum (NRS), purified by the same procedure as described above, was used as a control in all experiments.

For flow cytometry studies, the anti-ID antibody was affinity purified from serum using a mAb 36 Sepharose-4B column. Anti-ID antibodies were eluted with 3.5 M $MgCl_2$ and then dialyzed against PBS.

Preparation of Rabbit Antipeptide Antiserum. Peptide 104 (Synthesized by Bio-Synthesis, Inc., Denton, TX) was conjugated to keyhole limpet hemocyanin (KLH) using difluorodinitrobenzine (24). A rabbit was given a primary injection consisting of 100 μg p104-KLH mixed with an equal volume of CFA. Every 2 wk for 2 mo, animals were boosted with 100 μg p104-KLH plus an equal volume of IFA. Test bleeds were analyzed for specific antibody to p104 using an ELISA.

Preparation of Anti-ID $F(ab')_2$ Fragments. IgG was partially purified from anti-ID rabbit serum and NRS by ammonium sulfate precipitation. The IgG fractions were diluted 1:1 with 0.1 M phosphate buffer, pH 8, and passed through protein A–Sepharose CL-4B columns (Pharmacia Fine Chemicals, Piscataway, NJ). The columns were washed with 0.1 M phosphate buffer, pH 7.5, and the adsorbed antibody was eluted with 1 M acetic acid. The pH of the eluate was adjusted to 4.2 with 1 N NaOH, a solution of pepsin (1 mg pepsin/30 mg antibody) in 0.1 M acetate buffer pH 4.2 was added, and the mixture was incubated at 37°C for 16 h. The digestion was terminated by adjusting the pH of the solution to 7.6 with 1 N NaOH. Purification of $F(ab')_2$ fragments was accomplished by passage over a protein A–Sepharose CL-4B column. The $F(ab')_2$ fragments were dialyzed against PBS, and no detectable intact IgG was observed when assessed by SDS-PAGE analysis.

Preparation of Heterocross-linked Antibodies. $F(ab')_2$ fragments of either NRS (prebleed) or rabbit anti-ID were crosslinked with $F(ab')_2$ fragments of rabbit anti-DNP (25–27). Briefly, a fourfold molar excess of Succinimidyl-3-(2-Pyridyldithiol) propionate was added to either anti-ID $F(ab')_2$ (10 mg/ml) or NRS $F(ab')_2$ (25 mg/ml) in borate-buffered saline, pH 8.5 (BBS). Similarly, a threefold molar excess of Succinimidyl trans-4-(*N*-maleimidyl-mathyl) Cyclohexane-1-carboxylate (SMCC) was added to the rabbit anti-DNP $F(ab')_2$ (6 mg/ml) in BBS. After 30 min at room temperature, the anti-ID and NRS were brought to pH 4.5 and reduced, as described. These proteins then were passed through a Sepharose PD10 column in 0.1 M PO_4, 0.1 M NaCl, pH 7.5, and added to the SMCC-derivatized anti-DNP $F(ab')_2$. After induction for 4 h at 37°C, the heterocross-linked proteins were fractionated on a 1.6 × 90-cm vitragel AcA 34 column. Peaks containing $F(ab')_2$ (>100 kD) were pooled and concentrated. The crosslinked material was predominantly 150 kD in molecular mass (one anti-ID or NRS $F[ab']_2$ crosslinked to one anti-DNP $F[ab]_2$), as determined by SDS-PAGE. These crosslinked antisera were used to test for induction of cytotoxicity with targets that had been chemically modified by treatment with 1 M trinitrobenzoylsulfonate (25).

Binding Assay. The binding assay was performed by incubation of 100 μl of LGL (10^6 cells/ml) with $F(ab')_2$ fragments of NRS or anti-ID for 15 min at 22°C, followed by the addition of 100 μl of target cells (10^6 cells/ml), and centrifugation at 120 *g*

for 5 min at 22°C. The cells were then resuspended by gentle pipetting, and the percent of lymphocytes bound to target cells was determined by counting at least 200 lymphocytes in suspension.

Cytotoxicity Assay. Cytotoxicity assays were performed using a ^{51}Cr release assay as previously described (28). For experiments involving pretreatment of effector cells, 2×10^6 LGL/ml were incubated for 18 h at 37°C with various doses of whole antiserum, $F(ab')_2$ fragments or IL-2 (Cetus Corp., Emeryville, CA) and then washed before performing the 4-h ^{51}Cr release assay. In each experiment, a control was included using cells incubated in media alone. Three replicates were used for each experimental group, and the percent-specific lysis was calculated as: 100× [(total cpm in experimental wells) − (cpm in wells with target cells alone)/(total cpm incorporated into target cells)]. The percent control lysis was calculated as: 100× (percent experiment/percent control).

BLT Esterase Assay. BLT esterase activity was estimated using a microtiter assay (29). Briefly, 50 μl of sample was added to 100 μl of 1 mM dithiobis (2-nitrobenzoic acid) (DTNB) made up in PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.2. The reaction was initiated by adding 50 μl of 0.5 mM Nα-CBZ-L-Lys-thiobenzyl ester (BLT; Sigma Chemical Co., St. Louis, MO). The duration of the assay varied depending upon the length of time necessary for optimal color development. The rate of increase of absorbance at 410 nm was measured on a microplate reader (MR5000; Dynatech Corp., Alexandria, VA). Controls of sample and DTNB or DTNB and BLT were always performed to exclude nonspecific activities.

IFN-γ Assay. RIA of human IFN-γ (Centocor, Malvern, PA) was performed with culture supernatants obtained after treatment of 2×10^6 LGL/ml as described above.

Radiolabeling of Proteins. Intact $CD3^-$ LGL, T cells, and monocytes were surface labeled with ^{125}I using lactoperoxidase, which selectively labels only surface proteins (30). Briefly, 2×10^7 cells were washed and resuspended in 100 μl PBS. 1 mCi ^{125}I (16 mCi/μg; Amersham Corp., Arlington Heights, IL) was added, followed by 50 μl of lactoperoxidase (2 mg/ml PBS; Sigma Chemical Co.), and 20 μl 0.03% H_2O_2 in PBS at 25°C. After 5 min of incubation, 50 μl of lactoperoxidase and 20 μl of 0.03% H_2O_2 were added, and the mixture was incubated for another 5 min at 25°C. The reaction was terminated by adding 200 μl of 10^{-2} M potassium iodide in PBS. After extensive washing with PBS, the cells were lysed in 0.05 M Tris-HCl, pH 7.6, buffer containing 0.2 M NaCl, 0.005 M EDTA, 0.1 mM PMSF, 10 mM iodoacetamide, 1 μg/ml antipain, 1 μg/ml chymostatin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1% Triton X-100 (Sigma Chemical Co.). Proteins were immunoprecipitated from cell lysates with partially purified anti-ID serum or NRS, and protein A–Sepharose CL-4B. Immunoprecipitates were washed extensively with 0.05 M Tris-HCl, pH 7.6, 0.25 M NaCl, and 0.005 M EDTA, and 0.1% Triton X-100. Samples were analyzed by SDS-PAGE on 7.5% acrylamide gels (31).

Metabolic Labeling of Proteins. $CD3^-$ LGL were metabolically labeled with ^{35}S-methionine (Amersham Corp.) as previously described (32). The labeled cells were lysed and proteins immunoprecipitated as described above.

Flow Cytometry. 5×10^6 LGL in 50 μl HBSS containing 0.1% BSA and 0.1% NaN_3 were incubated for 30 min at 4°C with 5 μg/ml of anti-ID, anti-p104, or NRS. The cells then were washed twice with the same solution and labeled with a FITC-conjugated $F(ab')_2$ fragment of goat anti-rabbit IgG antiserum (Cappel Laboratories, West Chester, PA). In addition, binding of anti-CD3, anti-CD16, and CD56 (Leu19: Becton Dickinson & Co., Mountain View, CA) directly labeled with PE was analyzed using flow cytometry. Data were analyzed using FACScan® flow cytometer (Becton Dickinson & Co.).

Results

Identification of Immunoreactive Lymphocyte Subsets. To define the cell populations(s) that express the molecule to which the anti-ID and anti-p104 antisera react, two-color flow cytometry analysis was performed on PBL using anti-CD56-PE, anti-CD16-PE, anti-CD3-PE, NRS-FITC, $F(ab')_2$ anti-ID-FITC, and anti-p104-FITC. Fig. 1 denotes the results typically seen (representative of >25 donors) with various populations of lymphocytes. As shown in Fig. 1 *B*, panels *1–3*, using LGL-enriched lymphocytes (50%), most $CD56^+$ lymphocytes bound the anti-ID and, with less intensity, to the anti-p104 antibodies (panels *2* and *3*). Panel *4* shows that all of the $CD16^+$ cells also exhibited reactivity with the anti-ID antibodies. In contrast, $CD3^+$ lymphocytes in a total PBL population did not react with the anti-ID or anti-p104 antisera (panels *5* and *6*). However, in most donors, there was a small population of $CD3^-$, $CD56^+$ cells (both bright and dim staining) that did not react with the anti-ID or with the anti-p104. The anti-ID and anti-p104 did not react with human monocytes (representative of the cells found in quadrant 3 of panel *5*), fresh rat LGL, or human or rat T cells, and also did not react with the NK target cells, MOLT-4 and K562 (data not shown). These results suggested that the molecule to which the anti-ID and the anti-p104 bound was unique to human $CD3^-$ LGL.

Inhibition of Binding. Since the anti-ID antiserum bound to LGL, experiments were performed to determine whether this antiserum would block the binding of the LGL effectors to the tumor target cell. Inhibition of binding would suggest that the antigen(s) recognized by the antibody were involved in LGL-mediated target recognition. This hypothesis was tested by measuring the ability of $F(ab')_2$ fragments of anti-ID or NRS to inhibit binding of LGL to the NK-susceptible target cells K562 and MOLT-4. Since both LGL and K562 cells express Fc receptors, $F(ab')_2$ fragments of the anti-ID were necessary to avoid the possibility of nonspecific or Fc receptor–mediated reactions. When $CD3^-$ LGL were incubated with $F(ab')_2$ fragments of the anti-ID antiserum, a dose-dependent inhibition of conjugate formation between the LGL effectors and K562 or MOLT-4 target cells was observed (Table 1). A 1-μg dose of anti-ID inhibited the binding of LGL to K562 or MOLT-4 cells by 60% and 52%, respectively. These data suggested that the structure recognized by the anti-ID antiserum plays a role in the interaction between LGL and their target cells.

Specificity of the Anti-ID Antiserum. LGL are known to exhibit at least two mechanisms for cytotoxic activity. One lytic mechanism, ADCC, is mediated through the Fc receptor. However, the Fc receptor is not involved in NK cell–mediated lysis of tumor targets. If the anti-ID antiserum is recognizing a functionally important receptor involved in the antibody-independent lysis of tumor targets, it should be possible to inhibit NK cell–mediated lysis, but not ADCC, with

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B

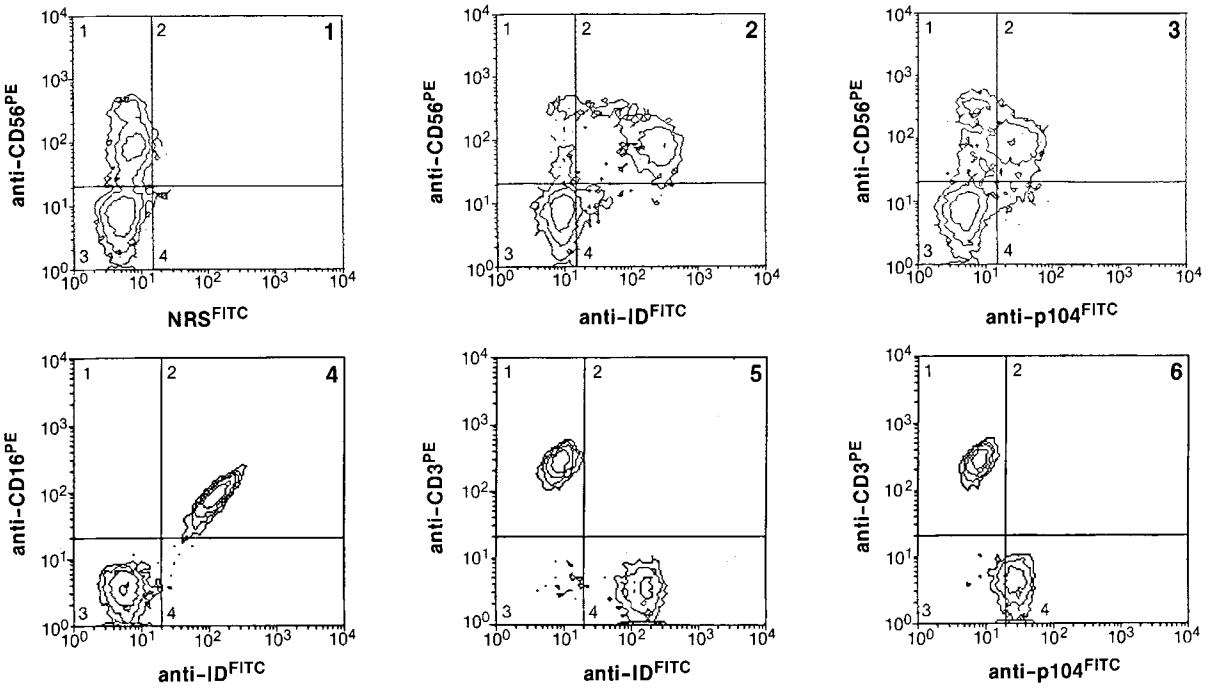

Figure 1. (*A*) The predicted amino acid sequence for the idiotype binding region (*bold type*) and the peptide sequence used in generating anti-p104 antisera (*underlined*). (*B*) Two-color flow cytometry studies. Low density lymphocytes containing 50% $CD3^-CD56^+$ and 50% $CD3^+CD56^-$ (panels *1–4*) or B cell–depleted lymphocytes containing 80% $CD3^+CD56^-$ and 20% $CD3^-CD56^+$ cells (panels *5* and *6*) were analyzed directly for their two-color expression of the rabbit anti-ID and lymphocyte markers. (Panel *1*) CD56/PE × NRS/FITC; (panel *2*) CD56/PE × anti-ID/FITC; (panel *3*) CD56/PE × anti-p104/FITC; (panel *4*) CD16/PE × anti-ID/FITC; (panel *5*) CD3/PE × anti-ID/FITC; (panel *6*) CD3/PE × anti-p104/FITC. Quadrant *1* (orange only); *2* (double positive), *3* (negative); and *4* (green only).

this antiserum. Therefore, the anti-ID antiserum was tested for its ability to inhibit the binding of the same LGL effector cells to the NK-resistant cell line (FeMX) that had been coated with rabbit antibody. Under these experimental conditions, the anti-ID did not block conjugate formation (Table I).

Since the anti-ID serum was found to inhibit antibody-independent binding of LGL to tumor cells, but not LGL-mediated antibody-dependent binding, we examined the ability of the anti-ID antiserum to inhibit NK activity vs. NK-mediated ADCC activity. The direct incubation of the $F(ab')_2$ fragments of the anti-ID with $CD3^-$ LGL and K562 target cells resulted in a dose-dependent inhibition of target cell lysis as measured by ^{51}Cr release, with 50% inhibition of cytotoxicity occurring at a dose of 20 μg/ml of anti-ID (Fig. 2 *A*). Higher doses of anti-ID ($\geq$80 μg/ml) resulted in complete inhibition of cytolytic activity in four of five donors tested. Control $F(ab')_2$ fragments of NRS, analyzed at these same concentrations, did not inhibit NK-mediated lysis. Similar results were observed when MOLT-4 cells were used as the targets (data not shown). Thus, the anti-ID selectively blocked NK activity and did not inhibit the FcR-dependent binding (Table 1) and lysis (Fig. 2 *A*) by $CD3^-$ LGL. In parallel experiments, other control antibodies that were reactive with adhesion molecules such as $CD11_a$ and ICAM lacked this specificity and blocked both forms of cytolysis (data not shown). Therefore, these data collectively demonstrate that the anti-ID can selectively inhibit the NK activity (both its ability to bind and kill) of a single effector cell population that also mediates ADCC. These data supported the hypothesis that the anti-ID was reacting with a cell surface molecule involved in NK-mediated lysis.

Because the rabbit antiserum contains antiallotype antibodies in addition to the anti-ID, it was critical to demonstrate that the active antibody in the rabbit antiserum was the anti-ID. Therefore, experiments were performed to examine the effects of anti-ID adsorption on antiserum binding (Table 1) and lysis (Fig. 2 *B*). When antiserum was depleted of anti-ID reactivity by mAb 36, an IgMκ, it no longer inhibited LGL-mediated binding (Table 1) nor lysis of target cells (Fig. 2 *B*). Parallel adsorption of the anti-ID with irrelevant control mAbs (an IgMλ and an IgMκ) did not remove these inhibiting activities. Treatment with control $F(ab')_2$

Table 1. *Inhibition of Binding between CD3⁻ LGL and NK-susceptible Targets by Anti-ID*

Targets	Percent Conjugates*	Percent inhibition			Absorbed with:‡		
		μg§	NRS	anti-ID	ID (IgM[κ])	MOPC (IgM[λ])	mAb35 (IgM[κ])
	± *SE*						
K562	59 ± 2	0.1	1	22‖	2	21‖	24‖
		1	3	60‖	2	61‖	64‖
		5	19‖	75‖	10	77‖	75‖
MOLT-4	34 ± 3	0.1	0	15‖	1	17‖	16‖
		1	5	52‖	3	51‖	54‖
FeMX (ADCC)¶	55 ± 4	0.1	0	0	ND	ND	ND
		1.0	1	2	ND	ND	ND
		5.0	2	1	ND	ND	ND

* Percent conjugates without antibodies (see Materials and Methods).
‡ Anti-ID sera adsorbed with Sepharose-4B-bound antibody. This loss of reactivity was seen with four (of four) affinity absorptions.
§ Refers to the micrograms of $F(ab')_2$ NRS or anti-ID used to inhibit conjugate formation.
‖ Significant inhibition at $p < 0.05$ (Student's T-test) compared to conjugates formed in the absence of antibodies.
¶ FeMX was coated with a rabbit antimelanoma serum and used as an ADCC target. FeMX is not susceptible to NK-mediated lysis and does not form conjugates with LGL in the absence of antibody.

fragments of NRS at the same dose also did not significantly inhibit conjugate formation.

Evidence that the Anti-ID Recognizes a Triggering Moiety. Previous studies have demonstrated that heterocross-linked antibodies containing one antibody against a receptor on the cytotoxic cell crosslinked to a second antibody that recognizes a target cell surface component can induce cytotoxic cells to lyse otherwise NK-resistant target cells. These studies have demonstrated that only triggering surface receptors like the TCR (CD3) and Fcγ receptors (CD16) on K/NK cells and monocytes are capable of inducing this redirected lysis (33). To determine whether the structure bound by the anti-ID was capable of promoting target cell lysis, anti-ID $F(ab')_2$ crosslinked with rabbit anti-DNP $F(ab')_2$ was added to $CD3^-$ LGL in the presence of DNP-modified YAC-1 mouse target cells that are resistent to lysis by human LGL. When the DNP-treated YAC-1 cells were exposed to the heterocross-linked antibodies on the $CD3^-$ LGL, lysis of the YAC-1 cells was observed (Fig. 3 *A*). In contrast, cytotoxicity was not observed when either non-DNP-modified YAC-1 targets or heterocross-linked anti-DNP $F(ab')_2$ × NRS $F(ab')_2$ were used. The heterocross-linked antibody alone was not cytotoxic against YAC-1. Since YAC-1 mouse target cells are resistant to lysis by human LGL, intact rabbit (Ig) anti-DNP was used as a positive control for ADCC activity (Fig. 3 *A*).

Induction of the LGL lytic mechanism can be monitored by the ability of an antibody against a triggering molecule to mediate reverse ADCC (6). When LGL were pretreated with either anti-ID or anti-p104 antisera before incubation with an FcR-positive NK-resistant Raji target, both antisera mediated reverse ADCC in a dose-dependent manner with the maximum lysis being 32% and 36% for the anti-ID and the anti-p104, respectively (Fig. 3 *B*). The maximum level of lysis obtained with NRS was 7%. Therefore, these results further supported the hypothesis that the anti-ID recognized a functional cytotoxicity-triggering molecule on the surface of LGL.

It has been reported elsewhere that both NK cells and T cells release serine esterases in response to perturbation of membrane receptors (34, 35). The data presented in Table 2 illustrate that incubation of LGL with $F(ab')_2$ fragments of either anti-ID or anti-p104 elicited LGL to release 10 and 42 U of BLT esterase, respectively (representative of three experiments). This level of activation by the anti-ID and anti-p104 was higher than the 2.0 U of BLT esterase observed for the positive control (LGL and K562 tumor target cells). In fact, the anti-p104-induced activation exceeded even the ADCC-positive control (20 U). However, the BLT esterase release induced by the normal rabbit serum did not exceed that induced by the negative controls, medium alone, or the stimulation of LGL with uncoated Raji cells. These data demonstrated that both antibodies were able to trigger serine esterase release in LGL in response to the interaction of the antibodies with their target cell ligand.

Another consequence of stimulating LGL with target cells or with IL-2 is the induction of IFN-γ secretion (6, 36). Therefore, if the anti-ID or anti-p104 antisera were reacting with a tumor recognition molecule, then treatment of LGL with these antisera might be expected to stimulate LGL to secrete IFN-γ. LGL were incubated with different doses of $F(ab')_2$

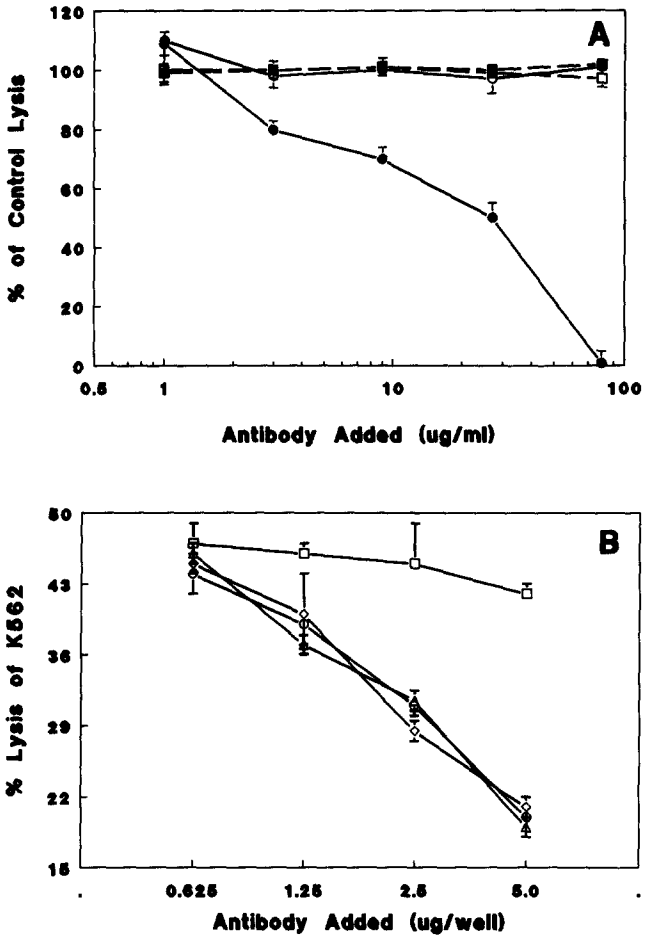

Figure 2. Specificity of anti-ID reactivity. (*A*) Inhibition of NK and ADCC cytotoxicity. NK (——) or ADCC (- - -) cytotoxicity was measured in the presence of anti-ID (NK, ●; ADCC, ■) and NRS (NK, ○; ADCC, □) and expressed as percent of control. Shown are results representative of five normal donors with an E/T ratio of 20:1. The mean percent lysis was 48.7% for NK activity and 65.4% for ADCC activity. (*B*) Effect of anti-ID adsorption on cytotoxicity against K562 cells. Cytotoxicity was measured in the presence of anti-ID (○); or after adsorption with the idiotype (□), (IgM[κ]); or two control antibodies, an IgM(λ) (◇) and an IgM(κ) (△). This graph shows results of a single representative experiment (of three). Percent lysis ± SE is shown using an E/T ratio of 20:1.

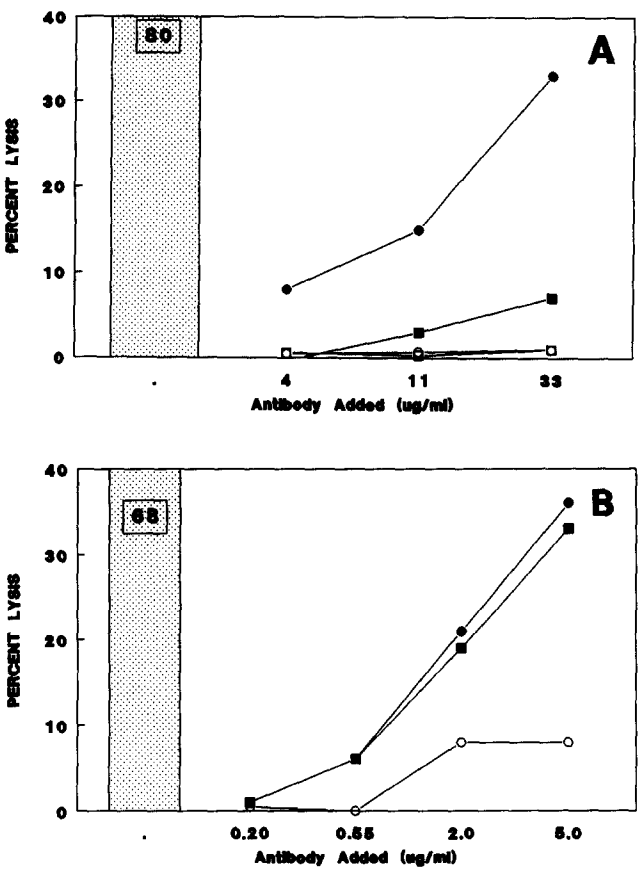

Figure 3. (*A*) Targeting of lysis with anti-ID containing hetero-crosslinked antibodies. LGL effector cells were pretreated with various concentrations of either ID/anti-DNP (● and ○) or NRS/anti-DNP (■ and □) hetero-crosslinked antibodies. These pretreated LGL were then incubated with unmodified YAC cells (○ and □) or with chemically modified YAC-1 cells (● and ■) at an E/T ratio of 30:1 for 4 h. Percent lysis was determined. Rabbit antibody (IgG) was used as a positive control for ADCC activity. (*B*) Reverse ADCC was determined by a 4-h ^{51}Cr release assay of an FcR^+ NK-resistant target, Raji. LGL were pretreated with varying amounts of NRS (○), anti-ID (■), or anti-p104 (●). The LGL were then incubated with Raji cells, and the percent lysis was determined. ADCC, using an antibody that reacts with the target only, was used as a positive control. Values represent specific lysis at an E/T ratio of 10:1. Differences of 5% are significantly different when analyzed using the student's paired *t* test ($p \leq 0.05$).

fragments of anti-ID, anti-p104, or NRS, or with IL-2. Supernatants were then assayed for IFN-γ levels using a RIA. The ability of IL-2 (1,000 U/ml) to stimulate LGLs to secrete IFN-γ (Table 3) was used as a positive control in each experiment. The amount of IFN-γ found in supernatants of anti-ID-treated LGL varied among donors; however, the induction of IFN-γ by the anti-ID was dose dependent. In both experiments, >0.02 μg/well (0.2 μg/ml) of anti-ID resulted in the induction of detectable levels of IFN-γ. Higher doses of anti-ID induced up to 300 U/ml of IFN-γ. In addition, absorption of the anti-ID antiserum with mAb 36 reduced the IFN-γ induction by >90%. The anti-p104 $F(ab')_2$ fragments (50 μg/ml) stimulated the LGL to secrete 45 U/ml of IFN-γ. These results demonstrated that both the anti-ID and anti-p104 antisera were capable of triggering the release of IFN-γ, in a manner similar to that previously reported when LGL were exposed to target cells or treated with IL-2. To ensure that anti-ID was not causing secretion of other lymphokines, levels of IL-2 production in anti-ID-treated LGL were also tested and no measurable secretion of IL-2 was detected (data not shown).

Biochemical Characterization of the Anti-ID- and Anti-p104-reactive Molecule(s). Biochemical analysis of the molecule(s) reactive with the anti-ID antiserum was performed using immunoprecipitation and SDS-PAGE gel electrophoresis. As shown in Fig. 4 *A*, the anti-ID immunoprecipitated an 80-kD molecule, a 110-kD molecule, and a 150-kD molecule from surface-labeled LGL under reduced (lane 2), or nonreduced

Table 2. *BLT-Esterase Release from CD3⁻ LGL Induced by Selected Antisera*

Treatment	Relative units of BLT-esterase released*
LGL + medium	≤0.5
LGL + K562 (NK activity)	2.0
LGL + Raji coated cells (ADCC)	20.0
LGL + untreated Raji cells	≥0.5
NRS $(Fab')_2$ – LGL + Raji	≥0.5
Anti-ID $F(ab')_2$ – LGL + Raji	10.0
Anti-p104 $F(ab')_2$ – LGL + Raji	42

Shown are results of a single representative experiment (of three).
* Units of BLT-esterase were determined after 4 h of incubation at 37°C. The relative units refer to the esterse activity obtained from 0.5×10^6 LGL and calculated at an OD of 0.1. 20 U represents ~ 30% of the total BLT-esterase that was present in the CD3⁻ LGL. K562 and Raji did not contain detectable BLT-esterase.

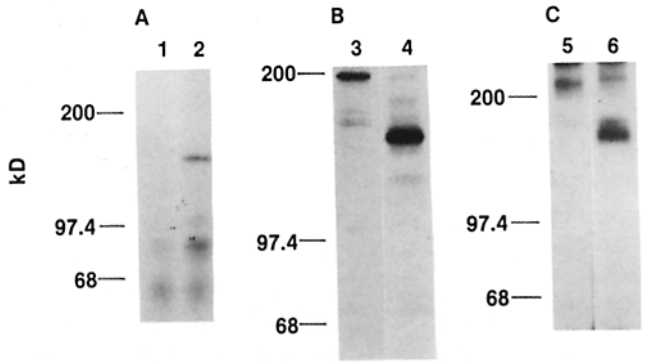

Figure 4. Biochemical characterization of proteins recognized by anti-ID and anti-p104 antisera. (*A*) ^{125}I CD3⁻ LGL surface-labeled cell lysates with NRS (lane *1*) or anti-ID (lane *2*). (*B*) ^{35}S-methionine LGL cell lysates were precipitated with NRS (lane *3*) or anti-p104 (lane *4*). (*C*) ^{125}I surface-labeled LGL cell lysates were immunoprecipitated with NRS (lane *5*) or anti-p104 (lane *6*). Gel electrophoresis using 7.5% SDS-PAGE was performed under reduced conditions for all gels followed by autoradiography. The molecular mass markers used were: myosin (H chain), 200 kD; phosphorylase B, 97.4 kD; BSA, 68 kD; OVA, 43 kD; α chymotrypsinogen, 25.7 kD.

conditions (data not shown). In contrast, immunoprecipitation of these molecules was not observed when NRS was used (lane *1*). The same proteins could not be immunoprecipitated from either T cells or monocyte membranes with the anti-ID antiserum (data not shown).

The predicted amino acid sequence for the ID binding region was delineated based on the cloning and sequencing of a novel gene from a human NK cell cDNA λgt11 expression library (Fig. 1 *A*) that was identified by the anti-ID antiserum (Anderson, S. K., S. Gallinger, R. Roder, J. L. Frey, H. Young, and J. R. Ortaldo, manuscript submitted for publication). A 20–amino acid sequence from the COOH end of the ID binding region (Fig. 1 *A*) was selected in an effort to obtain a more specific reagent that would aid in studying the relevance of the three proteins immunoprecipitated by the anti-ID antiserum to NK cell–mediated recognition and lysis. Since the anti-p104 serum exhibited similar functional reactivities as the anti-ID serum, biochemical analysis was performed in order to determine the molecular nature of the surface moiety that was recognized by the anti-p104 antiserum. LGL were either metabolically labeled or surface labeled and the cell lysates were immunoprecipitated with the anti-p104 antiserum. In LGL that were metabolically labeled, the anti-p104 antiserum immunoprecipitated a 150-kD molecule (Fig. 4 *B*). The recognition of the 150-kD molecule by the anti-p104 serum could be specifically inhibited by excess p104 (data not shown), and an irrelevant peptide did not inhibit the anti-p104-mediated immunoprecipitation (data not shown). Several other proteins of lower molecular masses were also immunoprecipitated by anti-p104 antibodies. However, the relationship between these proteins and their possible relevance to NK-mediated lysis is not yet understood. In similar experiments performed with the anti-p104 antiserum using surface-labeled LGL, only the 150-kD protein was immunoprecipitated by the anti-p104 serum (Fig. 4 *C*). These data suggest that the 150-kD protein was the functional LGL triggering molecule identified by both the anti-ID and anti-p104 sera.

Table 3. *Induction of IFN-γ Secretion by Anti-ID Serum*

Treatment	Dose antibody $F(ab')_2$	IFN-γ production Exp. 1*	Exp. 2*	Exp. 3‡
	μg/ml		*U/ml*	
None	–	<2	<2	<2
IL-2§	–	400	600	800
NRS	50			6
NRS	20	<2	<2	
NRS	2	<2	<2	
NRS	0.2	<2	<2	
Anti-ID	20	100	300	
Anti-ID	2	30	75	
Anti-ID	0.2	5	10	
Anti-p104	50			45

* In Exp. 1 and 2, the antisera was adsorbed with ID (mAb 36) and control IgMκ (mAb35). The ID resulted in removal of >90% of the IFN-γ induction (<5 and 10 U/ml in Exp. 1 and 3, respectively).
‡ Exp. 3 shows the results of a single representative experiment (of two) using anti-p104.
§ Used as positive control for IFN-γ production.

Discussion

To define the molecule(s) involved in target cell recognition by LGL, a rabbit anti-ID serum was prepared against

a mAb that recognized membrane glycoproteins on the NK-sensitive tumor cell target, K562. The rationale for this approach was that antibodies made against the antigen-combining site of the mAb, which reacted with a target cell moiety recognized by $CD3^-$ LGL, may mimic the configuration of the molecules on $CD3^-$ LGL that are involved in recognition of the target cell. Thus, the anti-ID serum should react with the target recognition structures on LGL. The approach used to determine whether the anti-ID serum was reacting with a potential tumor recognition molecule on NK cells was similar to that used to identify the TCR. Specifically, we designed experiments to assess whether the ligand for the anti-ID was unique to LGL and whether the anti-ID could block LGL-mediated binding and cytolysis. The present study demonstrates that the results obtained for LGL using this anti-ID serum parallel those criteria that were used to define TCR-mediated events. In particular, the anti-ID serum bound specifically to human $CD3^-$ LGL and blocked the ability of these LGL to bind to and lyse NK-sensitive tumor targets. In contrast, the anti-ID did not inhibit LGL-mediated ADCC. The anti-ID serum activated the lytic potential of LGL, as demonstrated by the ability of the antiserum to mediate reverse ADCC, and when heterocross-linked, the anti-ID could stimulate LGL to lyse NK-resistant tumor targets. In addition, the recognition of a surface moiety by both the anti-ID and anti-p104 (an antipeptide antibody made against a peptide sequence predicted to be part of the ID binding site) antibodies elicited the release of BLT esterase from LGL and induced LGL to secrete IFN-γ.

Biochemically, the anti-ID serum immunoprecipitated three proteins (80, 110, and 150 kD) from the surface of LGL. However, with the anti-ID reagent, we were not able to determine which protein(s) were relevant to NK-mediated recognition and lysis. The possibility exists that the NK recognition/triggering molecule used by NK cells is a complex of proteins much like that reported for the TCR. The relationship of these LGL proteins to each other and to LGL function is presently under investigation. The recent cloning of a unique gene using this anti-ID antiserum (Anderson, S. K., S. Gallinger, R. Roder, J. L. Frey, H. Young, and J. R. Ortaldo, manuscript submitted for publication) has made possible the development of a more specific antipeptide (anti-p104) serum. This reagent was demonstrated to have nearly identical functional reactivities as the anti-ID antiserum, but biochemically it recognized only the 150-kD protein on the surface of LGL. In addition to the 150-kD protein, other proteins of lower molecular masses were also recognized by the anti-p104 from metabolically labeled LGL. These proteins could represent different processed forms of the 150-kD molecule, molecules associated with the 150-kD protein, or proteins containing a crossreactive epitope. Experiments are in progress to further define the relationship between the lower molecular mass proteins and the 150-kD protein. Therefore, these data are consistent with the hypothesis that the 150-kD protein to which the anti-ID antiserum reacts was the important surface ligand, which potentially has receptor function for human $CD3^-$ LGL NK activity. The 80- and 110-kD molecules, which have different epitopes recognized by antibodies in the anti-ID serum not present in the anti-p104 serum, may represent associated entities that could comprise an NK recognition/triggering complex.

Several attempts have been made to determine if both the anti-ID and the anti-p104 recognize the same 150-kD molecule. Our most compelling evidence, though by no means conclusive, is based on the previously discussed biochemical data. Both antibodies immunoprecipitate a molecule with a molecular mass of 150 kD. In preliminary data, when the proteins are analyzed by two-dimensional gel electrophoresis, the 150-kD proteins immunoprecipitated by both antisera have the same pI of 5.0. In further supporting the hypothesis that both antibodies recognize the same protein, a β-galactosidase fusion protein, which was produced in *Escherichia coli* from the cloned gene, was found to block the immunoprecipitation of the 150-kD protein by the anti-ID antisera. Furthermore, based on the percentages of $CD56^+$ cells, at least 90% of the ID-reactive cells are also p104 reactive. Two-color FACS® analysis and crosscompetition for binding to LGL of the two antibodies have been performed, however, the data have been inconclusive. There are several explanations why these assays do not definitely address the issue of whether two antibodies are recognizing the same protein. For example, it is not possible to distinguish by two-color analysis or crosscompetition whether two different antibodies are recognizing different epitopes of the same molecule, whether they are recognizing two different molecules, or whether the binding of one antibody may be sterically interfering with the binding of the second antibody. Therefore, the most definitive proof that both antibodies are recognizing the same molecule is to analyze the amino acid sequence of the 150-kD protein immunoprecipitated by each antibody. These experiments are presently underway in our laboratory.

Based on the predicted amino acid sequence, the surface moiety recognized by the anti-ID antiserum has been shown to be different from the immunoglobulin supergene family and the integrin supergene family. This molecule contains several distinct structural features, including a cyclophilin-like domain, three positively charged domains, several histone-like domains, and several serine-rich domains. However, there is no homology with serine or tyrosine kinases or with protein phosphatases (Anderson, S. K., S. Gallinger, R. Roder, J. L. Frey, H. Young, and J. R. Ortaldo, manuscript submitted for publication). Because of the unique structure of this molecule and our finding that it plays an important role in the recognition and lysis of tumor cell by NK cells, this molecule may represent a novel class of receptors.

Several investigators have reported the development of mAbs that recognize surface molecules on fish LGL (37), rat LGL, and lymphokine-activated killer (LAK) cells (38), or human LGL (39, 40) and LAK cells (40). These molecules have been proposed to be involved in the activation and regulation of NK activity. However, none of these molecules are associated exclusively with NK activity. In contrast, preliminary studies of the p150 molecule we have identified demonstrated its expression on the cell surface of IL-2-activated lymphocytes by FACS® analysis, but no ability of the anti-ID serum to inhibit IL-2-activated killing (data not shown). This suggested

that the molecule(s) recognized by the anti-ID antiserum was important in target recognition and lysis by unstimulated NK cells, but that LAK cells recognized and/or lysed targets differently. This observation may partially explain the broader target cell spectrum for LAK-mediated cytolysis (41). Thus, since the anti-ID serum did not appreciably inhibit either LAK activity or ADCC, the ligand for the anti-ID appears to be a receptor specifically related to the mechanism of NK activity. Thus, the relationship between the 150-kD molecule (and the 110- and 80-kD molecules) identified in our studies and the proteins reported elsewhere remains unclear. None of the previously reported molecules (37–40) correlate with respect to size, function, and cellular distribution to the p150 molecule reported herein. To define whether a molecule is a true NK recognition receptor will require additional confirmation either by transfection of a full-length gene into cells lacking NK activity or by abrogation of expression of the proposed receptor in NK cell lines or clones with antisense cDNA vectors. However, based on the novelty of the gene that was cloned using the anti-ID antiserum, and the ability of this molecule to mediate reverse ADCC, trigger LGL to release BLT esterase, and induce LGL to secrete IFN-γ, when stimulated by the anti-ID or anti-p104, the 150-kD protein appears to represent an important molecule involved in the triggering of NK-mediated lytic function. Current studies are underway in our laboratories to determine if the 150-kD protein represents a biologically relevant NK cell recognition/triggering receptor.

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