CHEMILUMINESCENCE RESPONSE OF HUMAN NATURAL KILLER CELLS

I. The Relationship between Target Cell Binding, Chemiluminescence, and Cytolysis*

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Granulocytes and monocytes are known to phagocytose and kill numerous varieties of bacteria and parasites. This function is intimately associated with an early event called the "respiratory burst," which includes the generation of O_2^- and H_2O_2 (1). These and other highly reactive molecular species can easily be detected in the presence of luminol or other readily oxidized substrates by the emission of light (chemiluminescence), a phenomenon first described by Allen et al. (2) in reference to phagocytosing neutrophils. The exact mechanism of chemiluminescence is not yet understood (3) but appears related, in part, to species of singlet oxygen and electronically excited carbonyl groups that relax with ensuing light emission (2).

The respiratory burst is essential for chemiluminescence because it does not occur in resting cells, in stimulated cells from patients with chronic granulomatous disease, or in normal stimulated cells kept under anaerobic conditions (2, 4, 5). Chemiluminescence is also impaired in myeloperoxidase- (MPO)[1] deficient leukocytes or by treatment of cells with heme enzyme inhibitors such as sodium azide (2, 4–6). In addition, phagocytosis-associated chemiluminescence in normal cells is attenuated by both superoxide dismutase (SOD) and catalase (5–7) and is correlated in time with the formation of O_2^- and H_2O_2 (8). These and other data indicate that chemiluminescence is dependent on the availability of O_2, the activity of the initial oxidase responsible for O_2^- formation, the presence of O_2^- and H_2O_2, and the activity of MPO.

Microbes are thought to be killed directly by cell-derived H_2O_2 and possibly O_2^- and certain free radicals. Tumor cells, on the other hand, are destroyed in part by natural killer (NK) cells (10) in a process that does not involve phagocytosis, much like nonphagocytic killing of tumor cells by macrophages. It has previously been shown that murine macrophages use H_2O_2 in their cytolytic mechanism (11), as do human neutrophils (12), but very little is known about the cytolytic mechanism in NK cells. In this paper, we show that the NK-target cell interaction results in an

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[1] *Abbreviations used in this paper:* ADCC, antibody-dependent cellular cytotoxicity; NK, natural killer lymphocyte; PBS, phosphate-buffered saline; SOD, superoxide dismutase.

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immediate burst of chemiluminescence. The degree of chemiluminescence is directly correlated with the sensitivity of the target cells to NK-mediated binding and cytolysis. In addition, the generation of chemiluminescence is dependent upon the degree of activation because interferon-boosted NK cells show increased chemiluminescence. It is postulated that those cellular reactions that generate chemiluminescence could be important in the cytolytic pathway of human NK cells.

Materials and Methods

Cell Lines. All cell lines were maintained by continuous in vitro culture and were free of mycoplasma at the time of testing. K562 is a human erythroleukemic cell line derived from the pleural effusion of a patient with chronic myelogenous leukemia in terminal blast crisis. K562-induced clones A4, G8, and B were derived from the K562 cell line grown in 1 mM sodium butyrate or 0.1 mM haemin and maintained in culture for several months after cloning. Erythroid induction was monitored by gross cytoplasmic changes (increased granularity), benzidine (Lephene's) staining, increases in quinhydrone production and hemoglobin synthesis, and increased amounts of glycophorin (13). P815 is a mastocytoma induced in DBA/2 mice with methylcholanthrene. YAC is a Moloney leukemia virus-induced T cell lymphoma of A/Sn mice origin. L5178Y is a lymphoma induced in DBA/2 mice with methylcholanthrene. Chang is a human hepatocarcinoma. Human embryonic fibroblasts (HEF) were in passage 10 of primary cell culture. MeWo is a human melanoma.

Effector Cells. Heparinized venous blood was treated for 30 min at 37°C with carbonyl iron (type E; A. D. McKay, Darien, CT; 100 mg per 10 ml blood). The blood was then passed over a magnet followed by centrifugation (1,000 *g*, 10 min) on Ficoll-Hypaque (specific gravity 1.077 g/cm^3). The mononuclear cell band was collected and incubated for 1 h at 37°C on plastic culture dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) precoated with autologous plasma. The nonadherent lymphocytes contained <1% monocytes as assessed by phagocytosis of antibody-coated ox erythrocytes or latex particles, by immunofluorescent staining with Mo2, a monoclonal antibody specific for the entire human monocyte population (14), or by cytochemical stains for esterase and peroxidase. In control studies, carbonyl iron did not affect monocyte detection by any of these methods. Contamination with granulocytes was <0.1% as judged in Giemsa cytocentrifuge preparations and immunofluorescent staining with a monoclonal antibody specific for human granulocytes.

Monocytes. Human peripheral blood mononuclear cells (5 ml at 3–4 × 10^6 cells/ml) were incubated for 1 h at 37°C on 60-mm plastic culture dishes (Falcon Labware, Div. of Becton, Dickinson & Co.) that had been precoated for 15 min at 37°C with autologous plasma. Nonadherent cells were collected, and the dishes were then thoroughly washed with warm medium. Strongly adherent cells were recovered by trypsinization and incubated for 6 h at 20°C in culture medium to allow "recovery." Adherent cells consisted of >95% monocytes as assessed by nonspecific acid esterase and peroxidase staining and immunofluorescence with monoclonal Mo2 antibody.

Granulocytes. Human granulocytes were collected by unit gravity sedimentation of the erythrocyte-granulocyte pellet obtained after Ficoll-Hypaque removal of mononuclear cells.

Percoll Fractionation. Discontinuous Percoll (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ) density gradients were made essentially as described by Timonen and Saksela (15). Briefly, the top layer consisted of 40% Percoll (vol/vol) diluted in medium and adjusted to 285 mosmol/kg H_2O with 10-fold-concentrated phosphate-buffered saline (PBS) using an Osmette osmometer (Fisher Scientific Co., Pittsburgh, PA). The refractive index of this top fraction was adjusted to 1.3432, and subsequent fractions were established by increasing the density of the Percoll by 2.5% increments. The gradient was loaded with ~10^8 monocyte-depleted lymphocytes and centrifuged at 550 *g* for 30 min at 20°C. The lymphocyte bands were collected and washed twice in medium, whereas the pellet, consisting mainly of erythrocytes, was discarded. Lymphocyte fractions along with unfractionated cells were tested in a 2-h ^{51}Cr release assay against K562 to determine which fraction contained peak NK activity.

Immunofluorescence. Lymphocytes (5 × 10^5 in 0.1 ml RPMI containing 1 mM NaN_3 and 10% fetal calf serum) were incubated for 1 h at 0°C with a 1/500 dilution (20 μg/ml) of monoclonal

HNK-1 antibody (IgM-kappa) (16), washed twice, and incubated for a further 1 h at 0°C with a 1/10 dilution of rhodamine-labeled [F(ab')$_2$ fragment] goat anti-mouse IgM (N. L. Cappel Laboratories Inc., Cochranville, PA), as described by Abo and Balch (16). The cells were washed twice and prepared as wet mounts. 500 cells were counted using a fluorescent microscope (E. Leitz, Federal Republic of Germany). In some experiments, the cells were stained with monoclonals OKT3 (Ortho Pharmaceutical, Raritan, NJ) (1/50) or Mo2 (1/100) (generously provided by Dr. R. Todd and Dr. S. Schlossman, Boston, MA) followed by a 1/10 dilution of fluorescein isothiocyanate-labeled (F(ab')$_2$ fragment) sheep anti-mouse IgG (H and L chain specific; N. L. Cappel Laboratories Inc.).

Conjugate-forming Cell Assay. Monocyte-depleted lymphocytes from the highly NK-enriched fraction of Percoll gradients were mixed with a fivefold excess of tumor cells, centrifuged at 150 *g* for 5 min at 20°C, and then stored on ice. Pellets were gently resuspended, and the number of lymphocytes binding to tumor cells was determined in duplicate by counting 200 lymphocytes in a hemacytometer.

Cytolytic Assays. Target cells labeled with ^{51}Cr (sodium chromate) were mixed with varying numbers of effector cells in 150 μl in V-bottomed microplates (Linbro Chemical Co., Hamden, CO) in triplicate samples. The microplates were centrifuged at 150 *g* for 5 min and then incubated in a 37°C humidified 5% CO_2 incubator for 4 or 18 h. After incubation, the plates were again centrifuged for 10 min, and 100-μl samples were removed for counting in a gamma counter (Beckman Instruments, Inc., Fullerton, CA). The percentage cell-mediated lysis (percent CML) was calculated as follows: percent CML = 100 × ([cpm test − cpm medium])/([cpm max − cpm medium]). The cpm (max) was determined by counting a 100-μl aliquot of resuspended target cells. Counts per minute (medium) was determined in wells containing targets only with no effectors added.

Lytic Units. Lytic units (LU) were calculated from effector titration curves, and 1 LU was defined as the number of effector cells required to achieve 20% lysis. LU/10^7 is the number of LU in 10 million effector cells.

Interferon Treatment. For cytolytic assays, 5 million monocyte-depleted lymphocytes were incubated (10^6 cells/ml) for 1 h at 37°C with 200 U/ml of purified fibroblast interferon (10^8 U/mg protein; generously provided by C. Tan, University of Calgary) or with medium as control and washed twice before use. For chemiluminescence assays, 5 × 10^5 NK-enriched, Percoll fraction cells were incubated for 1 h at 25°C with 200 U/ml of interferon, washed, and then used.

Glutaraldehyde Treatment. Glutaraldehyde (Sigma Co., St. Louis, MO) was made up to 0.20 and 0.25% from two different batches. 30 × 10^7 K562 target cells or 10^6 NK-enriched Percoll fraction lymphocytes were added to 10 ml of a glutaraldehyde solution, incubated at 37°C for either 1 or 2 h, washed three times with PBS, and stored at 4°C in PBS until used.

Chemiluminescence. One-half or 1 million lymphocytes from the NK-enriched Percoll fraction were dark-adapted for 30 min at 20°C in 0.8 ml of 10% luminol (3-aminophthalhydrazide; Eastman Kodak Co., Rochester, NY)-saturated FCS in plastic scintillation vials. 10 million target cells or medium alone was added in 0.1-ml volumes and mixed under red light illumination. Chemiluminescence was measured at ambient temperature (20°C) in an LKB liquid scintillation counter (LKB Instruments, Inc., Rockville, MD) in the out-of-coincidence mode (17). Counts per minute were recorded at 5-s intervals. Luminol alone gave a background of ~35 × 10^3 cpm, and Percoll-fractionated lymphocytes with luminol had a background of 50 × 10^3 cpm. 10^7 target cells tested with luminol gave no chemiluminescence above that of the luminol alone.

Plasma Membrane Vesicles. 10^8 tumor cells were disrupted in a nitrogen bomb, and plasma membrane vesicles were isolated on sucrose density gradients as described previously (18).

Statistical Analysis. The differences between experimental groups were determined using a two-tailed Student's *t* test. The significance of linear regressions was determined using the Fisher exact test.

Results

Chemiluminescence Follows NK Target-Effector Interaction. NK cells were enriched by Percoll gradient fractionation and dark-adpated in the presence of luminol. The

addition of K562 tumor cells led to a rapid chemiluminescence response that was directly proportional to the concentration of effector cells or targets, as shown in Fig. 1. In the presence of a fixed number of 10^7 target cells, 10^6 NK-enriched lymphocytes generated a peak chemiluminescent response of 2×10^6 counts over the 10-min assay period (Fig. 1, left panel). The reason for the decline in the chemiluminescence response when the effectors were increased to 2×10^6 is not known but could conceivably arise because of the release of SOD or scavengers from cells killed at this higher effector/target ratio. When the effector cells were held constant at 0.5×10^6, a near maximum chemiluminescence response was observed upon addition of 10^7 intact K562 cells (Fig. 1, right panel). In subsequent assays, therefore, we used 0.5×10^6 Percoll fractionated effector cells and 10^7 intact target cells.

Plasma membrane vesicles of K562 were less able to induce chemiluminescence at high concentrations compared with intact K562 cells, whereas low numbers of vesicles (0.01×10^6 cell equivalents, 0.028 μg protein) generated markedly more chemiluminescence than intact K562 cells. Because each K562 cell yields many plasma membrane vesicles, one would expect intact cells to be more limiting than vesicles, as we observed. These results imply that a given number of hits is necessary to trigger the NK cells regardless of particle size. Vesicles prepared from an NK-insensitive target, P815, failed to induce any detectable chemiluminescence response.

In these experiments, we used mixtures of targets and effectors without knowing exactly which cell type was generating the oxygen radicals. Therefore, we used glutaraldehyde fixation to inactivate one of the two partners. The NK-enriched

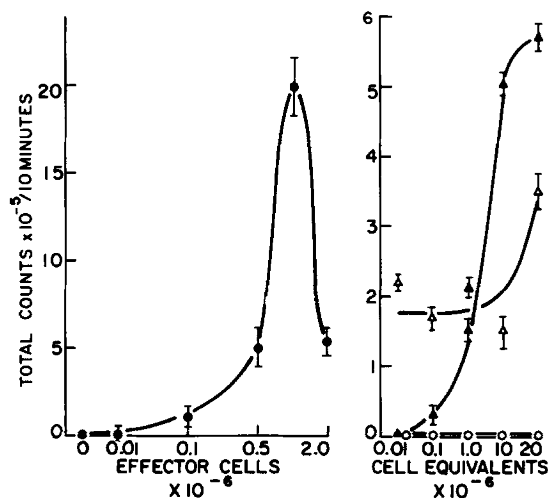

FIG. 1. Effector/target titrations in the chemiluminescence assay. Monocyte-depleted peripheral blood lymphocytes were separated on Percoll density gradients, and the fraction highest in NK-mediated cytotoxicity was selected for further study. Left panel: varying numbers of these lymphocytes were mixed with 10^7 K562 cells at a time 0 in the presence of luminol, and cpm were recorded every 5 s on an LKB liquid scintillation counter in the out-of-coincidence mode (●). Right panel: varying numbers of intact K562 cells were mixed with 5×10^5 NK-enriched Percoll fraction lymphocytes (▲). Cell-free plasma membrane vesicles were prepared from K562 (△) and P815 (○) cells and mixed with 5×10^5 NK-enriched Percoll fraction lymphocytes. 10^7 cell equivalents of plasma membrane vesicles contained 28 μg of protein. Target cells or vesicles alone did not generate chemiluminescence. Values represent mean total cpm above background ± SE over a 10-min period in triplicate samples. This experiment was repeated three times with similar results.

Percoll fraction was fixed for 1 h at 37°C in 0.20% glutaraldehyde (lot 2) and then washed extensively in PBS. When 10^7 K562 cells were added, no response above background was detected in the chemiluminescence assay, as shown in Fig. 2. 51 ± 2% of the fixed NK cells formed conjugates with K562 in a single-cell assay, compared with 49 ± 4% of unfixed NK cells. Therefore, the glutaraldehyde treatment did not affect the ability of NK cells to bind to the target cells. The same glutaraldehyde treatment of monocytes completely abolished the chemiluminescence response to the chemotactic peptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (data not shown). Therefore, glutaraldehyde treatment seems to abolish the ability of cells to generate chemiluminescence in general.

Glutaraldehyde fixation of K562 target cells under mild conditions preserved the target structure because these cells were almost as efficient as unfixed K562 cells in competing with ^{51}Cr-labeled targets in a cytolytic assay, as shown in Table I. Conjugate formation with Percoll fraction 2 lymphocytes was also within normal range, and the fixed target cells induced almost as much chemiluminescence (97%) as unfixed K562 cells. These results show that it is the NK-enriched lymphocytes that respond to K562 cells by generating chemiluminescence, whereas the target cells do not respond to the effectors. It is also noteworthy that K562 cells subjected to harsh

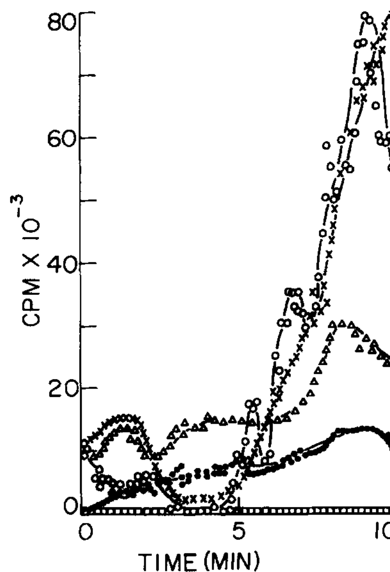

FIG. 2. Induction of chemiluminescence with glutaraldehyde-fixed target cells. Monocyte-depleted peripheral blood lympocytes were separated on Percoll density gradients, and the fraction highest in NK-mediated cytotoxicity was selected for further study. 5 × 10^5 NK-enriched Percoll fraction lymphocytes were mixed with 10^7 untreated K562 cells (○) or 10^7 K562 cells fixed using glutaraldehyde treatment protocols a (×), b (△), or c (●) at time 0 in the presence of luminol, and cpm were recorded every 5 s on an LKB liquid scintillation counter in the out-of-coincidence mode. 10^7 K562 cells were treated with (*a*) 0.20% glutaraldehyde (lot 2) for 1 h at 37°C; (*b*) glutaraldehyde (lot 2) at a concentration of 0.25% for 1 h at 37°C; or (*c*) glutaraldehyde (lot 1) at a concentration of 0.25% for 2 h at 37°C. NK-enriched Percoll fraction lymphocytes were treated with glutaraldehyde 0.20% (lot 2) for 1 h at 37°C and washed three times with PBS. 5 × 10^5 of these fixed lymphocytes were mixed with 10^7 K562 cells, and chemiluminescence was measured as above (□). Values represent cpm above background. The area under each curve varied <3% in repeat samples. This experiment was repeated three times with similar results.

TABLE I

Target Cell Competition and Chemiluminescence Induced with Glutaraldehyde-fixed K562 Target Cells

Treatment*	Competitor/ target ratio for 20% inhibition‡	Cells required for 20% inhibition × 10^{-4}‡	Percent conjugates§	Chemiluminescence‖
				Percent control
Untreated K562	3/1	6 ± 2	58 ± 4	100 ± 2
Glutaraldehyde-treated K562 (*a*)	7.5/1	15 ± 3	53 ± 3	97 ± 3
Glutaraldehyde-treated K562 (*b*)	42/1	84 ± 5	22 ± 2	74 ± 1
Glutaraldehyde-treated K562 (*c*)	143/1	286 ± 10	9 ± 3	20 ± 3

* Treatment protocol: (*a*) 10^7 K562 cells were treated with 0.20% glutaraldehyde lot 2 for 1 h at 37°C. (*b*) 10^7 K562 cells were treated with 0.25% glutaraldehyde lot 2 for 1 h at 37°C. (*c*) 10^7 K562 cells were treated with 0.25% glutaraldehyde lot 1 for 2 h at 37°C.

‡ Unfixed or glutaraldehyde-fixed K562 cells were added at varying ratios (from 0.3/1 to 20/1) to a 4-h ^{51}Cr release assay of NK-enriched Percoll fraction lymphocytes and K562 target cells (ratio, 50/1). Values are expressed as the competitor/target ratio or mean competitor cell number ± SE required to inhibit lysis by 20% in triplicate samples. The actual percent lysis was 70%.

§ Percoll fraction-2 cells were mixed with K562 cells at a 1:5 ratio, and the percent lymphocytes bound to K562 (conjugates) was determined after counting 200 lymphocytes. Values represent the mean of triplicate samples ± SE.

‖ Chemiluminescence of NK-enriched Percoll fraction lymphocytes was measured over a 10-min period, and the area under the curve was integrated. Values are expressed as the mean percent chemiluminescence induced by glutaraldehyde-fixed K562 cells compared with the unfixed control ± SE in triplicate samples. The control value was 4×10^5 total counts over 10 min.

fixation conditions were less able to bind to the NK cells or compete in a cold target competition assay and were also less able to induce chemiluminescence. As shown in Table I, there was a direct correlation between the preservation of the target structure, as measured in competition assays or conjugate formation, and the ability to induce chemiluminescence. We then asked whether this relationship would hold in a survey of different tumor cell lines of varying NK susceptibility.

The Degree of Chemiluminescence Is Correlated with the NK Sensitivity of the Tumor Cell and the State of NK Activation. We chose a panel of nine target cell types that varied in their sensitivity to NK-mediated killing and macrophage killing for further study. The most highly NK-enriched Percoll fraction was tested in a standard conjugate-forming assay with each of these targets. As shown in Table II, there was a direct relationship between conjugate formation and sensitivity to NK-mediated cytolysis. A 4-h chromium release assay was used to evaluate sensitivity to NK-mediated cytolysis using Ficoll-separated, iron-treated, and plastic-nonadherent peripheral blood lymphocytes as effectors. In the case of relatively insensitive targets, we used an 18-h cytolytic assay. The data were calculated as LU/10^7 effectors and then standardized to the K562 target that was always run in parallel. As shown in Table II and Fig. 3, K562 clones A4, B, and G8 each had a marked decrease in ability to trigger chemiluminescence (30–48% of control), which correlated with a reduction in susceptibility to NK-mediated cytolysis (21–33% of control) and conjugate formation (22% of control) compared with the K562 parent. These clones are selectively resistant to NK cells and were derived from K562 cells induced to differentiate by growth in sodium butyrate (13).

L5178Y, P815, and YAC were all relatively insensitive to human NK killing, even

TABLE II

Chemiluminescence Induced by Target Cells of Varying NK Sensitivity

Target cell	Chemiluminescence*	Percent conjugates‡	NK cytolysis§	Monocyte cytolysis‖
	Percent control		Percent control LU	Percent control LU
K562-uninduced parental	100 ± 2	40 ± 3	100 ± 5	0.72 ± 0.1
K562-induced clone A4	30 ± 4	8 ± 2	21 ± 3	0.76 ± 0.2
K562-induced clone B	48 ± 1	9 ± 1	33 ± 2	0.71 ± 0.1
K562-induced clone G8	48 ± 3	8 ± 2	33 ± 3	0.70 ± 0.1
L5178Y	15 ± 1	2 ± 1	1 ± 1	100 ± 4
P815	20 ± 2	0 ± 0	5 ± 2	0.36 ± 0.1
YAC	20 ± 3	3 ± 1	4 ± 1	6.9 ± 1
HEF	164 ± 4	55 ± 4	250 ± 10	NT*
Chang	29 ± 1	12 ± 1	20 ± 1	NT

* Chemiluminescence of NK-enriched Percoll fraction lymphocytes in response to various target cells was measured over a 10-min period, and the area under the curve was integrated. The data for each target are expressed as the mean percent ± SE of total cpm stimulated by K562 cells over the same time period in triplicate samples. The actual control value was 3.2×10^6 total counts over 10 min. Each experiment was done at least three times and some were done five times.

‡ Conjugates were formed with an excess of target cells using the NK-enriched Percoll fractionated lymphocytes. Values represent the mean ± SE in triplicate determinations.

§ Monocyte-depleted peripheral blood lymphocytes were tested in a ^{51}Cr release assay for 18 h against L5178Y, P815, and YAC and were tested for 4 h for all the other targets. LU were calculated at 20% lysis. LU values for K562 target cells run in parallel assays for 4 h (900 LU/10^7) or 18 h (1,100 LU/10^7) were normalized to 100%, and the other values were compared with it. Values represent the mean ± SE of triplicate samples.

‖ Monocytes (>95% esterase and $Mo2^+$) isolated by plastic adherence were tested in an 18-h ^{51}Cr release assay against each target. Values for L5178Y target cells (330 LU/10^7) run in parallel were normalized to 100% and the others compared with it. Values represent the mean ± SE of triplicate samples. Chemiluminescence vs. conjugates: $r = 0.93$; $P < 0.01$. Chemiluminescence vs. NK cytolysis: $r = 0.98$; $P < 0.001$. NK cytolysis vs. conjugates: $r = 0.98$; $P < 0.001$.

* Not tested.

in an 18-h assay (1–5% of K562 control), and they were likewise deficient in inducing chemiluminescence (15–20% of K562 control) (Table II, Fig. 3, panel b). Human embryonic fibroblasts (HEF) were more sensitive than K562 to NK-mediated killing (250% of K562 control), and they also induced a higher amount of chemiluminescence (164% as compared with K562 control) (Fig. 3, panel c). Chang, on the other hand, is intermediate in sensitivity to NK-mediated cytolysis (20% of K562 control) and induced the generation of an intermediate (29%) chemiluminescence response (Fig. 3, panel d).

These data indicate that the amount of chemiluminescence generated in response to the NK target-effector interaction is directly related to the sensitivity of the target cell to NK-mediated binding and lysis. It was then relevant to ask whether the degree of NK activation for cytolysis also influenced the level of chemiluminescence.

Interferon is known to have a profound boosting effect on human NK cell cytolytic activity (19). As shown in Table III, interferon pretreatment of NK-enriched lymphocytes significantly increased the level of cytotoxicity against MeWo and K562 target cells by 150% and 50%, respectively. The amount of chemiluminescence generated by interferon pretreated cells was increased in parallel by 50% for MeWo (Fig. 4) and 20% for K562 targets (Table III). These data show that agents such as interferon,

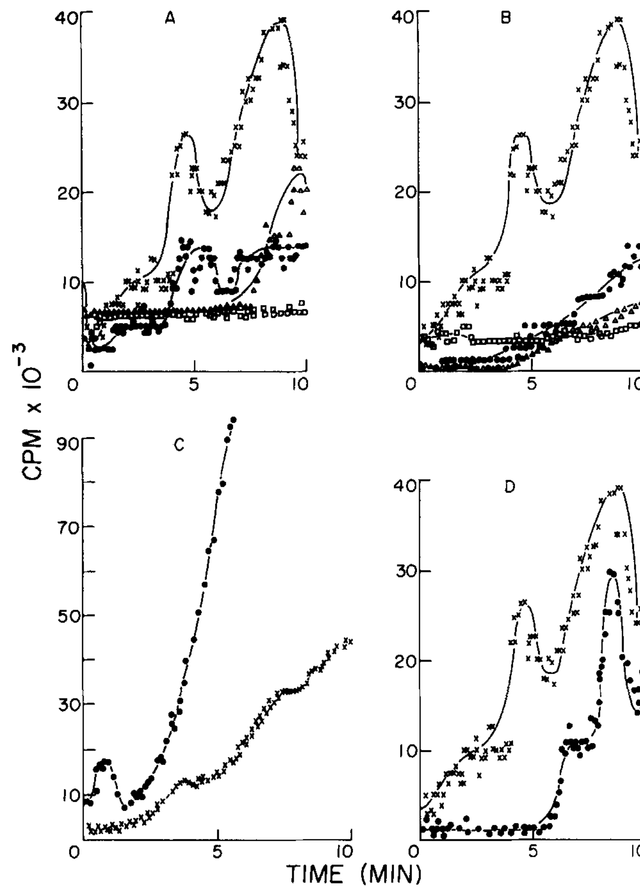

FIG. 3. Induction of chemiluminescence by cell lines of varying susceptibility to NK cytolysis. Monocyte-depleted peripheral blood lymphocytes were separated on Percoll density gradients, and the fraction highest in NK-mediated cytotoxicity was selected for further study. 5×10^5 of these lymphocytes were mixed with 10^7 target cells at time 0 in the presence of luminol, and cpm were recorded every 5 s on an LKB liquid scintillation counter in the out-of-coincidence mode. Values represent cpm above background. The area under each curve varied <4% in repeat samples. The experiments were repeated 5, 4, 3, and 3 times for panels A, B, C, and D, respectively, with similar results. Panel A shows uninduced parental K562 cells (×), induced K562-clone A4 cells (□), induced K562-clone G8 cells (●), and induced K562-clone B cells (Δ). Panel B shows K562 cells (×), YAC cells (●), L5178Y cells (Δ), or P815 cells (□). Panel C shows K562 cells (×) or human embryonic fibroblast cells (●). HEF peak at 180×10^3 cpm at 10 min. Panel D shows K562 cells (×) or 10^7 Chang cells (●).

which boost NK activity, also boost the generation of chemiluminescence. We then asked whether other effector cell types could generate chemiluminescence.

Chemiluminescence and Cytotoxicity by Monocytes. Macrophages and granulocytes are known to generate chemiluminescence and, therefore, we felt it was necessary to prove that contamination with these cell types was not responsible for the chemiluminescence we attribute to NK cells. Preparation of the effector cells for our chemiluminescence assay included Ficoll separation, iron and magnet treatment, plastic adherence depletion, and Percoll density gradient fractionation.

Less than 0.1% of the cells recovered were granulocytes, as judged by Giemsa

TABLE III

Chemiluminescence in Interferon-activated NK Cells

Target	Treatment*	Chemiluminescence‡	P value§	NK Cytolysis‖	P value
		Percent control		Percent control LU	
MeWo	No interferon	100		100	
MeWo	With interferon	150	<0.01	250	<0.001
K562	No interferon	100		100	
K562	With interferon	120	<0.02	150	<0.01

* Lymphocytes were treated with 200 U of beta (fibroblastic) interferon for 1 h and washed or left untreated.

‡ Chemiluminescence was measured over a 10-min period, and the area under the curve was integrated. The percent chemiluminescence is derived by total cpm treated lymphocytes per total cpm untreated lymphocytes × 100. Variations in repeat samples were <3%. The actual values for untreated MeWo and K562 were 1.75×10^5 and 9.05×10^5 total cpm over 10 min.

§ Monocyte-depleted peripheral blood lymphocytes were interferon-activated or untreated and were tested in a 4- or 18-h ^{51}Cr release assay against K562 and MeWo target cells, respectively. $LU/10^7$ values for untreated lymphocytes were 1,300 for K562 and 110 for MeWo. These values were normalized to 100% for comparison with treated lymphocytes run in parallel.

‖ *P* values were determined by a Student's *t* test for data from three repeat experiments per group.

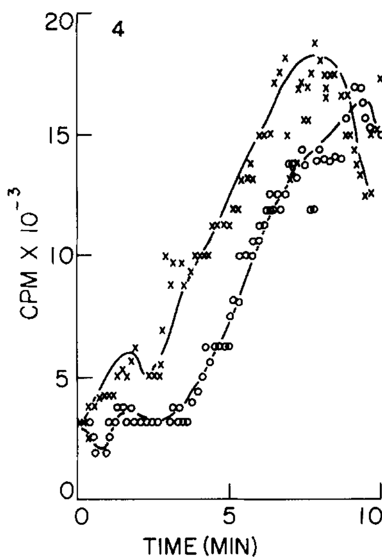

FIG. 4. Induction of chemiluminescence in interferon-activated lymphocytes. Monocyte-depleted peripheral blood lymphocytes were separated on Percoll density gradients, and the fraction highest in NK-mediated cytotoxicity was selected for further study. 5×10^5 of these lymphocytes were treated for 1 h (at 25–37°C) with 200 U of β interferon (fibroblastic) and washed. 5×10^5 interferon-treated (×) or untreated (○) lymphocytes were mixed with 10^7 MeWo cells at time 0 in the presence of luminol, and cpm were recorded every 5 s on an LKB liquid scintillation counter in the out-of-coincidence mode. Values represent cpm above background. The area under each curve varied <3% in repeat samples. This experiment was repeated three times with similar results.

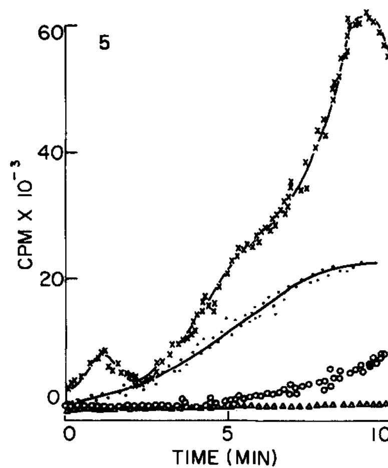

FIG. 5. The chemiluminescent response of granulocytes and monocytes to NK-sensitive and resistant targets. Monocytes (>95% esterase-positive, peroxidase-positive, and $Mo2^+$) were isolated by plastic adherence. Monocyte-depleted peripheral blood lymphocytes were separated on Percoll density gradients, and the fraction highest in NK-mediated cytotoxicity was selected for further study. Granulocytes (>99% polymorphonuclear and 99.5% positive for monoclonal antibody against PMN) were isolated from leukocyte pellets after depletion of mononuclear cells on Ficoll-Hypaque. 5×10^6 NK-enriched lymphocytes (×), 10^6 monocytes (○), or 10^5 granulocytes (△) were mixed with 10^7 K562 cells at time 0 in the presence of luminol. 10^5 monocytes were also mixed with 10^7 L5178Y cells (●). Counts per minutes were recorded every 5 s on an LKB liquid scintillation counter in the out-of-coincidence mode. Values represent cpm above background. 10^5 monocytes or granulocytes alone gave no response above background. The area under each curve varied <3% in triplicate samples, and differences between curves × and ○, or ● and △, were significant at the $P < 0.001$ level. This experiment was repeated three times with similar results.

staining and morphology or by immunofluorescent staining with a monoclonal antibody specific for human granulocytes. 60% of the Percoll-enriched cells expressed the NK-specific marker HNK-1 (16), and 40% expressed T3, a T cell marker (20). Staining with Mo2, a specific monoclonal antibody against human monocytes (14), or with nonspecific esterase and peroxidase revealed <1% $Mo2^+$ cells in non-Percoll fractionated cells and <0.1% $Mo2^+$ cells out of 1,000 cells counted in the Percoll fraction used for chemiluminescence. When the plastic adherence step was left out, the Percoll fraction used for chemiluminescence was found to have 10–25% $Mo2^+$ cells. In each of these cases where the Percoll fraction had 0.1, 10, or 25% $Mo2^+$ cells, there was no difference in the generation of chemiluminescence (data not shown). Conversely, monocyte-enriched adherent cells (90–95% acid esterase-positive and $Mo2^+$) were assayed alone in the chemiluminescence assay at 10^5 cells per vial, which is equivalent to 20% macrophage contamination in our standard Percoll fraction of 5×10^6 cells per vial. As shown in Fig. 5, 10^5 monocytes per vial gave only a minimum response to the K562 stimulus that was 7% of that in the NK-enriched Percoll fraction stimulated with K562 ($P < 0.001$). 10^5 granulocytes gave no response. As a positive control, 10^5 granulocytes generated a peak chemiluminescence response of 120×10^3 cpm at 40 min in response to 3×10^8 opsonized *Escherichia coli* per ml. 10^4 monocytes or 10^4 granulocytes also gave no response alone or when added to Percoll fraction 2 cells (data not shown).

Finally, some targets, such as L5178Y, showed poor killing by NK cells (1% of

K562 control) and a corresponding inability to induce chemiluminescence (15% of K562 control), as shown above in Table II. In contrast, L5178Y was the most sensitive monocyte target (Table II) and also induced four- to fivefold more chemiluminescence than K562 in monocytes ($P < 0.001$) (Fig. 5). YAC followed this same pattern, and repeat experiments, using monocytes isolated on Percoll gradients (21) rather than by adherence, gave similar results (data not shown).

Discussion

These results show that target-effector contact induces the NK cell to trigger a chemiluminescence response. The target structures recognized by the NK cell may play a role in this rapid activation because the amount of chemiluminescence produced is directly proportional to the ability of a given target cell line to bind to the NK cell and be lysed.

In the present study, we examined a panel of nine different target cell lines and found a striking correlation ($r > 0.93$) between target-effector conjugate formation, induction of chemiluminescence in NK cells, and susceptibility to NK-mediated cytolysis. The highly sensitive human tumor cell line K562 was shown to lose its sensitivity to NK-mediated binding and cytolysis when induced to differentiate with butyrate (Table II). This change in NK sensitivity was highly selective because sensitivity to antibody-dependent cell-mediated cytotoxicity and monocyte-mediated cytolysis was not altered in differentiated clones (13). K562 clones A4, B, and G8 also triggered only 30–48% of the chemiluminescence induced by the K562 parent cells. This correlation between ability to form target-effector conjugates, susceptibility to NK-mediated lysis, and the amount of chemiluminescence generated held for all the other targets as well. The relatively NK-insensitive targets YAC, P815, and L5178Y formed few target-effector conjugates and induced only a low level of chemiluminescence. Highly NK-sensitive cell targets (HEF) were able to induce a corresponding high amount of chemiluminescence, and cell lines (Chang) of intermediate NK sensitivity induced an intermediate amount of chemiluminescence.

Glutaraldehyde fixation of K562 cells was shown to preserve the target structure to varying degrees, as determined by the ability of these fixed cells to compete with unfixed K562 cells in a cold target inhibition assay or by direct visualization of target-effector conjugates. Here too, we found a strong correlation ($r > 0.95$) between ability to compete for the NK receptor and ability to trigger chemiluminescence (Table I, Fig. 2). These results are compatible with those of Bubbers and Henney (22), who showed that tumor cell targets treated with 0.3% glutaraldehyde lost their ability to specifically bind alloimmune killer T cells, whereas targets treated with 0.15% glutaraldehyde retained this ability. The data presented here suggests that it is the target structure binding to the NK receptor itself that may be the trigger for chemiluminescence. Intact target cells were not necessary because cell-free membrane vesicles made from NK-sensitive but not NK-insensitive targets also induced high levels of chemiluminescence. The observation that glutaraldehyde-fixed effectors bound to intact target cells but did not generate chemiluminescence suggests that (*a*) the effector is responding to the target and not the converse, and (*b*) binding is a necessary but not sufficient stimulus for chemiluminescence.

The state of NK activation was also found to influence the generation of chemiluminescence upon target-effector interaction. As shown in Fig. 4 and Table III,

interferon pretreatment of NK-enriched lymphocytes caused a concomitant increase in chemiluminescence induced by MeWo and K562 and a subsequent increase in NK-mediated cytolysis of both targets. The degree of augmentation of both chemiluminesence and cytolysis in interferon-boosted vs. -nonboosted effectors was greater with MeWo target cells compared with K562. Preliminary results have shown that oxygen radical scavengers block the interferon boosting of NK-mediated cytolysis (unpublished observation).

The responder cell that generates chemiluminescence after target-effector interaction is most likely an NK cell for a variety of reasons. First, the Percoll-enriched fraction contained 60% of cells bearing the NK-specific differentiation antigen HNK-1 described by Abo and Balch (16). The remaining 40% of the cells were predominantly $T3^+$ T cells (20); and few, if any, monocytes (<0.1%) bearing the Mo2 marker (14) or characteristic cytochemical markers were detected. We have also shown (23) that removal of $HNK\text{-}1^+$ cells with monoclonal anti-HNK-1 and complement abolishes the chemiluminescence response, and Percoll fractions consisting of nearly 100% T cells have no chemiluminescence response. It should be emphasized that monocytes do not express HNK-1 (16), and, conversely, the Mo2 marker is apparently specific for the entire human monocyte population (14) and does not react with NK cells (unpublished observation). Furthermore, purified populations of monocytes or granulocytes generated very little chemiluminescence against K562 at cell concentrations derived on the assumption of a 20% monocyte contamination of our NK-enriched populations (Fig. 5). Deliberate contamination of our NK-enriched Percoll fraction did not alter the chemiluminescence induced by K562. Therefore, a monocyte or granulocyte contamination of our NK-enriched effectors cannot account for the observed chemiluminescence.

At high cell numbers, monocytes were capable of generating a chemiluminescence response to those tumor cell lines that were sensitive to monocyte-mediated cytolysis but resistant to NK-mediated lysis (Fig. 5). Nonphagocytic killing of tumor cells by murine macrophages has been shown to involve H_2O_2 production (11). These results suggest that NK recognition and binding to target determinants triggers the NK cell to induce chemiluminescence. The underlying basis of this chemiluminescence response may involve O_2^- production in part because NK-target interaction also reduces extracellular cytochrome c at a rate of 200 pmol/min per 10^6 cells.[2] Preliminary results show that SOD (≤100 μg/ml, 280 U/ml) but not catalase (100 μg/ml, 1,180 U/ml) or denatured SOD block most (80%) of the NK-mediated chemiluminescence, cytochrome c reduction, and cytolysis with no effect on target-effector binding (unpublished data). Although O_2^- and possibly other highly reactive molecules might be involved in the chemiluminescence response shown here, further work is necessary to determine whether they activate other events in the cytolytic pathway or play a direct role in delivering the lethal hit.

Summary

The binding of tumor cells or fetal fibroblasts to human natural killer (NK) cells led to a rapid chemiluminescence response within seconds of target-effector interaction. The degree of chemiluminescence was dependent on the concentration of NK-

[2] Roder, J. C., S. L. Helfand, J. A. Werkmeister, R. McGarry, T. J. Beaumont, and A. Duwe. Oxygen intermediates are triggered early in the cytolytic pathway of human NK cells. *Nature (Lond.)*. In press.

enriched lymphocytes or target cells, and plasma membrane vesicles from K562 also induced a chemiluminescence response. Mild glutaraldehyde treatment of effector cells abrogated their ability to generate chemiluminescence, whereas K562 target cells treated in the same way were almost fully able to induce a chemiluminescence response to NK-enriched lymphocytes. These results show a directionality of response with NK as the responders and tumor cells as the stimulators. A survey of eight different tumor cell lines and fetal fibroblast lines revealed a striking correlation ($r > 0.93$, $P < 0.001$) between the ability of a given line to bind to NK-enriched lymphocytes, induce chemiluminescence, and to be lysed. Three differentiated sublines of K562 grown in butyrate and cloned induced little chemiluminescence compared with the K562 parent, and they were selectively resistant to NK-mediated binding and cytolysis. In addition, treatment of K562 cells with higher concentrations of glutaraldehyde for longer periods led to varying degrees of target antigen preservation, as measured in cold target competition assays and in conjugate formation. The degree of NK target antigen preservation correlated directly with the ability of the cells to induce chemiluminescence ($r > 0.95$). The degree of NK activation was also important because interferon-pretreated effectors generated more chemiluminescence upon stimulation with K562 or MeWo targets. Monocytes or granulocytes did not contribute to the chemiluminescence induced by NK-sensitive targets. Some NK-resistant tumor cell lines were sensitive to monocyte-mediated cytolysis and also induced chemiluminescence in monocytes but not NK cells. These results show that the target structures recognized by the NK cell may play a role in NK activation because the degree of chemiluminescence was directly proportional to the ability of a given target cell line to bind to the NK cell and to be lysed.

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