Mechanisms of Antibody-dependent Cellular Cytotoxicity

THE USE OF EFFECTOR CELLS FROM CHRONIC GRANULOMATOUS DISEASE PATIENTS AS INVESTIGATIVE PROBES

PAUL KATZ, CHARLES B. SIMONE, PIERRE A. HENKART, and ANTHONY S. FAUCI,
Clinical Physiology Section, Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, and the Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

ABSTRACT The present study characterized the antibody-dependent cellular cytoxicity (ADCC) of leukocyte effector cells (neutrophils, lymphocytes, and monocytes) from normal subjects and from chronic granulomatous disease (CGD) patients. CGD phagocytic cells (neutrophils and monocytes) had depressed ADCC activity against antibody-coated human erythrocyte (HRBC) targets in suspension cultures indicative of abnormal intracellular postphagocytic killing. However, when phagocytosis was prevented by using a monolayer of antibody-coated HRBC targets, CGD monocytes, neutrophils, and lymphocytes exhibited normal ADCC activity. Similarly, antibody-coated HRBC targets in suspension could be lysed normally by CGD effector cells when phagocytosis was inhibited by the addition of in vitro colchicine. Extracellular lysis of autologous antibody-coated lymphoid cell targets in suspension was mediated normally by CGD effector cells.

Thus, standard ADCC against HRBC targets in suspension is predominantly indicative of postphagocytic killing and, as such, is dependent upon a normal postphagocytic respiratory burst of oxidative metabolism which is deficient in CGD neutrophils and monocytes. Extracellular killing of sensitized targets does not appear to be dependent upon the generation of hydrogen peroxide (H_2O_2) and/or superoxide (O_2^-) and is normal in CGD neutrophils and monocytes. Hence, by employing CGD leukocytes as investigative probes in ADCC, fundamental mechanisms of intracellular vs. extracellular expression of cytotoxicity have been delineated.

Received for publication 6 July 1979 and in revised form 4 September 1979.

INTRODUCTION

Antibody-dependent cellular cytotoxicity (ADCC)[1] involves the destruction of sensitized target cells through the interaction of target-specific antisera with effector cell surface Fc receptors (1). Depending on the type of assay, antibody, target cells, and effector cells employed, the distinct ADCC activities of subpopulations of human leukocytes may vary. Thus, ADCC activity has been reported for neutrophils, monocytes, and Fc receptor-bearing (FcR^+) T and non-T lymphocytes (1). ADCC against antibody-coated human erythrocyte (HRBC) targets in suspension culture has generally been felt to be mediated predominantly by phagocytic cells (i.e., neutrophils and monocytes) (2). As such, this assay system may primarily reflect postphagocytic intracellular lysis of target cells. Conversely, extracellular lysis is the predominant mode of cell killing when antibody-coated nucleated nonerythroid target cells are employed (1).

The relative contribution of intracellular and extracellular events in ADCC in suspension culture are unclear. Additionally, the mechanisms of these two different modes of cytolysis as well as their potential interrelationship are presently speculative. In an attempt to further delineate the potential mechanisms of ADCC, the present study employed leukocytes from normal subjects and from patients with chronic granulomatous disease (CGD) as effector cells in ADCC assays against HRBC targets in suspension, autologous lymph-

[1] *Abbreviations used in this paper*: ADCC, antibody-dependent cellular cytotoxicity; ALS, antilymphocyte serum; CGD, chronic granulomatous disease; E:T, effector to target; FcR^+, Fc receptor-bearing; HRBC, human erythrocyte; 7S EA, bovine erythrocytes coated with IgG; TNP, trinitrophenol.

oid cell targets in suspension, and HRBC targets in monolayers. Phagocytic cells from CGD patients are deficient in NADPH oxidase activity and consequently lack phagocytosis-induced hydrogen peroxide (H_2O_2) and superoxide (O_2^-) generation (3–5). We therefore used effector cells from these patients as investigative cellular probes in an attempt to delineate the importance of H_2O_2 and O_2^- and related mechanisms in intracellular and extracellular ADCC activity.

METHODS

Subjects. Peripheral venous blood was obtained from 20 normal adult donors. Four patients with CGD were studied: three male ages 11 (patient 4), 13 (patient 1), and 21 (patient 2), and one female (patient 3), age 15. These patients fulfilled the previously described criteria for the clinical and laboratory diagnosis of CGD (6, 7). Each of the patients studied showed no nitroblue tetrazolium reduction upon stimulation with latex particles (8). In addition, all patients were incapable of generating O_2^- as monitored by modification of the ferricytochrome *c* reduction assay (9) using ionophore A23187 and the chemoattractant f-Met-Leu-Phe (1 μM) (gift of Dr. Elliott Schiffmann, National Institute of Dental Research, National Institutes of Health) plus cytochalasin B (5 μg/ml) (10) (data kindly supplied by Drs. Bruce E. Seligmann and John I. Gallin, National Institute of Allergy and Infectious Diseases, National Institutes of Health). All patients were well and free of infection at the time of study and none were receiving medication except patient 1 who had been receiving daily low-dose sulfisoxazole for several years.

Cell suspensions. Mononuclear cell suspensions (lymphocytes and monocytes) were obtained by standard Ficoll-Hypaque (Ficoll, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.; Hypaque, Winthrop Laboratories, New York) density centrifugation of anticoagulated peripheral venous blood (11). Lymphocytes were obtained after Ficoll-Hypaque centrifugation of peripheral venous blood; which had been monocyte-depleted by carbonyl iron treatment (12). Such treatment produced lymphocyte populations containing <1% monocytes by morphology. Purified suspensions of neutrophils were obtained by dextran sedimentation of either erythrocyte-neutrophil buttons obtained after Ficoll-Hypaque separation (13) or whole blood anticoagulated with EDTA (14).

Identification of mononuclear cell subpopulations. Mononuclear cells with surface receptors for the Fc portion of immunoglobulin (Ig)G were identified by modification of a described method (15). Briefly, FcR^+ mononuclear cells were identified by their ability to form rosettes with bovine erythrocytes coated with rabbit antibovine erythrocytes coated with IgG (7S EA) after incubation at 37°C for 20 min. The proportion of FcR^+ neutrophils was determined by a method similar to that described (16). Neutrophil suspensions were incubated at room temperature with bovine 7S EA for 20 min. Rosette-forming neutrophils and mononuclear cells (those binding three or more erythrocytes) were identified under phase-contrast microscopy. Cells with ingested 7S EA were identified on Wright's-stained cytocentrifuge preparations of suspensions of mononuclear cells or neutrophils that had been incubated for 45 min with 7S EA. Noningested 7S EA were hypotonically lysed with saline before enumeration of phagocytic cells. Bovine 7S EA was standardly employed rather than human 7S EA for the measurement of phagocytosis because bovine erythrocytes are smaller than human erythrocytes and appear to be phagocytosed more readily. However when human 7S EA was used instead of bovine 7S EA, comparable results were obtained (data not shown). T cells were identified by their ability to form spontaneous rosettes with sheep erythrocytes. Surface Ig-bearing cells (i.e., B cells) were identified through the use of fluoresceinated goat $F(ab')_2$ anti-human Ig. Lymphocytes and neutrophils were identified on Wright's-stained cytocentrifuge preparations. Monocytes were identified by morphology on Wright's-stained cytocentrifuge preparation and by nonspecific esterase staining.

Target cells. Fresh HRBC from an A^+ or an AB^+ donor were used in all studies. After washing with phosphate-buffered saline, 10^6 HRBC were labeled with 300–400 μCi of ^{51}Cr (Amersham Corp., Arlington Heights, Ill.) at 37°C for 1–2 h, washed, and resuspended in RPMI-1640 or balanced salt suspension Hepes media. In some experiments, ^{51}Cr-labeled HRBC were modified with trinitrophenol (TNP) by reacting with 10 mM trinitrobenzenesulfate at pH 7.4 for 15 min at 37°C. Fresh autologous human lymphoid cells to be used as targets in ADCC were obtained by Ficoll-Hypaque centrifugation and labeled with ^{51}Cr as described above in the presence of 10^{-1} stock dilution of rabbit IgG antilymphocyte serum (ALS).

Antibodies used in ADCC assays. Five different types of antisera were employed in these studies. (*a*) For target cell monolayer preparations, plastic surfaces were coated with affinity-purified sheep IgG anti-TNP antibody (0.5 mg/ml in phosphate-buffered saline). TNP-conjugated target cells were then allowed to attach to the coated plastic surfaces.[2] (b) For purified lymphocyte and neutrophil-mediated ADCC, affinity-purified rabbit IgG anti-TNP antibody (0.5 mg/ml in PBS) was employed at a final concentration of 25 μg/microtiter well. (c) Human IgG anti-blood group B antibody at a final dilution of 1:100 was used to coat the HRBC targets for the monocyte-mediated ADCC (17). (d) Rabbit IgG anti-HRBC antisera (N. L. Cappel Laboratories Inc., Cochranville, Pa.) was used in other experiments for mononuclear cell- and neutrophil-mediated ADCC activity against HRBC targets at a concentration of 10^{-1} stock dilution. (e) Rabbit IgG ALS was purified by DEAE-Sephacel (Pharmacia Fine Chemicals, Inc.) column fractionation of serum from rabbits immunized with human mononuclear cells. This antisera was used at a final concentration of 10^{-1} of stock dilution.

Cytoxicity assays. Two basic types of ADCC assays were employed: those with target cells in suspension and those with target cells in monolayer. For suspension ADCC against rabbit IgG antibody-coated HRBC targets, 10^6, 10^5, or 10^4 effector cells in RPMI-1640 were pipetted into wells of Microtest II tissue culture plates (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). Rabbit IgG anti-HRBC antisera were added to each well in a final concentration of 10^{-1} stock dilution and 100 μl of ^{51}Cr-labeled HRBC targets (10^4 cells) were added to the wells resulting in a final volume of 200 μl per well. Effector:target (E:T) ratios of 100:1, 10:1, and 1:1 were used in all experiments. Spontaneous release was determined by placing 10^4 targets into wells with 100 μl RPMI-1640 in the absence of effector cells. Cultures were incubated at 37°C in 5% CO_2 in air at 100% humidity for 18 h. Plates were then centrifuged and one-half the supernatant (100 μl) was removed and counted in an automatic gamma counter. Percent cytotoxicity (or percent ^{51}Cr release) was determined by the formula: (supernatant counts per minute − spontaneous release counts per minute/total counts − (2× spontaneous release counts per minute) × 2. In this formula the supernatant counts per minute is multiplied by two because the counts per minute of only one-half

[2] Simone, C. B., and P. A. Henkart. Manuscript in preparation.

of the total supernatant is determined. In all cases, spontaneous release was <13%.

Suspension assays using TNP-modified HRBC targets were performed slightly differently. Briefly, 0.7-1.0 × 10^6 ^{51}Cr-labeled TNP-HRBC were pipetted into individual wells to which either 1 × 10^6 neutrophils, 1 × 10^6 mononuclear cells (for monocyte-specific ADCC), or 2 × 10^6 lymphocytes were added. As required, 10 μl of the appropriate antibody was added to each well. Plates were incubated at 37°C for 3 h and the radioactivity determined for one-half the supernatant. For these experiments, spontaneous release was always <10%.

For assays using target cells in monolayer, microtiter wells were coated with 25 μl of sheep IgG anti-TNP antibody for 15 min at 25°C and washed with water. 100 μl of a 4% suspension of TNP-modified intact ^{51}Cr-labeled HRBC were then added to each well. After centrifugation, each well was washed five to seven times with balanced salt suspension Hepes and adherent target cells were observed under phase microscopy to verify a dense monolayer of targets. The desired number of effector cells and the appropriate antibody were then added to each well. After incubation at 37°C for 3 h, plates were centrifuged and 100 μl of supernatant were removed from each well and the determinations of ^{51}Cr release were performed.

For suspension ADCC against ^{51}Cr-labeled rabbit IgG ALS-coated autologous lymphoid cell targets, 10^6, 10^5, or 10^4 effector cells were pipetted into microtiter wells containing 10^4 target cells. Spontaneous release was determined as described above and was <20% in all cases. Cultures were incubated at 37°C in 5% CO_2 in air at 100% humidity for 4 h. Plates were then centrifuged and the percent cytotoxicity (or percent ^{51}Cr release) was determined.

In some experiments, colchicine (Sigma Chemical Co., St. Louis, Mo.) was added to the mixture of effector and target cells at the initiation of culture.

Statistical methods. Data were compared by the Student's two-tailed *t* test.

RESULTS

ADCC activity against HRBC targets in suspension. In suspension assays, unfractionated mononuclear cells from normal individuals mediated significant ADCC activity against HRBC targets coated with a 10^{-1} dilution of rabbit IgG anti-HRBC antisera at an E:T ratio of 100:1 (Fig. 1). However, mononuclear cells from CGD patients had markedly diminished ADCC activity against these same targets under the same conditions ($P < 0.001$ for all) (Fig. 1). This depression of CGD ADCC responses remained constant regardless of the E:T ratio (Fig. 2) or the concentration of rabbit IgG anti-HRBC antibody employed (data not shown). Additionally, when this activity was assayed at 1, 2, 4, 6, or 12 h after initiation of culture, CGD mononuclear cell effector capabilities remained depressed relative to normal mononuclear effector cells (data not shown). When mononuclear cell suspensions from normal subjects were depleted of monocytes to <1% by passage over Sephadex G-10 columns (Pharmacia Fine Chemicals, Inc.), ADCC activity against HRBC targets in suspension fell from 45±6 to 7±4% ($P < 0.001$). Presumably the remaining ADCC activity was mediated by nonphagocytic, FcR$^+$ lymphocytes (i.e., T cells and null cells). When mononuclear cell suspensions from patient 2 were depleted of monocytes, ADCC against HRBC targets fell insignificantly from 16±3 to 6±4% ($P < 0.1$). However, this value for killing by monocyte-depleted CGD mononuclear cells is comparable to that mediated by normal monocyte-depleted mononuclear cells ($P < 0.2$). Thus, nonphagocytic, FcR$^+$ CGD lymphocytes are capable of extracellular lysis of antibody-coated HRBC comparable to that observed with normal lymphocytes. When the monocyte-specific ADCC of patient 2 was additionally determined against human IgG anti-blood group B, antibody-coated, TNP-modified HRBC targets in suspension, 12% specific ^{51}Cr release was observed as compared to 54% ^{51}Cr release mediated by monocytes from a normal subject.

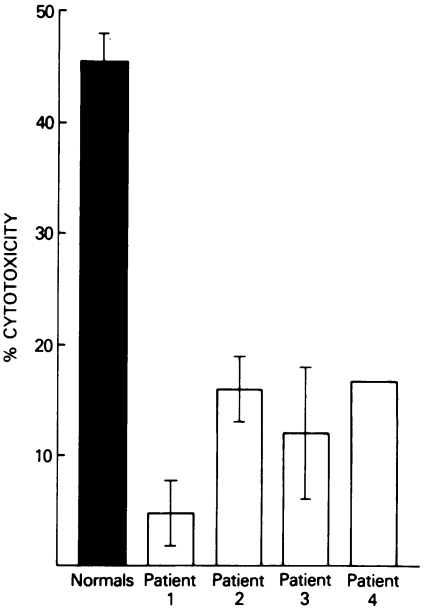

FIGURE 1 ADCC activity of normal and CGD mononuclear cells against rabbit IgG anti-HRBC-coated HRBC targets in suspension. At 100:1 E:T ratios, CGD mononuclear effector cells had markedly diminished ADCC activity when compared to normal mononuclear cells. Normal results were compiled from 11 separate experiments with different subjects. CGD patients 1–3 were studied on three separate occasions, whereas patient 4 was studied once.

With rabbit IgG anti-HRBC antisera, normal neutrophils induced substantial ^{51}Cr release from antibody-coated HRBC targets in suspension (Fig. 3). However, CGD neutrophils were incapable of significant target cell lysis when compared to normal subjects ($P < 0.001$ for all) (Fig. 3). This depression of neutrophil-mediated ADCC was constant regardless of E:T ratios, (Fig. 4) dilution of antisera, or time of culture interval (0.5–6 h). When neutrophils from patient 2 were additionally assayed against antibody-coated, TNP-modified HRBC target cells in suspension, 14% ^{51}Cr release was observed compared to 68% ^{51}Cr release induced by neutrophils from a normal subject.

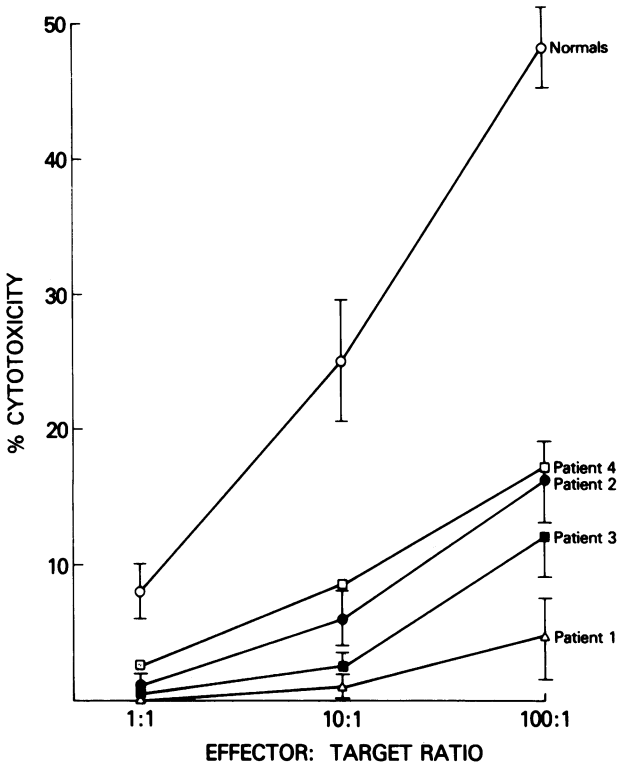

FIGURE 2 ADCC activity of normal and CGD mononuclear cells against rabbit IgG anti-HRBC-coated HRBC targets in suspension at 100:1, 10:1, and 1:1 E:T ratios. CGD mononuclear cells had markedly diminished ADCC activity at all ratios when compared to normals. Normal results were compiled from 11 separate experiments with different subjects. CGD patients 1–3 were studied on three separate occasions; whereas patient 4 was studied once.

The precise mechanisms(s) of ^{51}Cr release from phagocytes after intracellular HRBC lysis is uncertain but probably involves both spontaneous release from viable phagocytic cells and release from dead or dying effector cells. To rule out the possibility that CGD neutrophils or monocytes did not release intracellular ^{51}Cr at the same rate as normal cells, neutrophils and monocytes from three normal subjects and from patients 2 and 3 were labeled with ^{51}Cr and the spontaneous release determined after 1, 2, 4, 6, 12, and 18 h in culture. Additionally the viabilities of these unlabeled cells were determined by trypan blue exclusion at these same intervals. At each time interval, CGD phagocytic cells released ^{51}Cr and had the same viability as phagocytic cells from normal subjects (data not shown). Therefore, the depression of intracellular killing by CGD phagocytic cells cannot be attributed to an artifact of either a slow rate of release from effector cells of intracellular ^{51}Cr from ingested, killed targets or from differences in the viability of effector cells.

ADCC activity against HRBC targets in monolayer. All the above experiments were performed with target

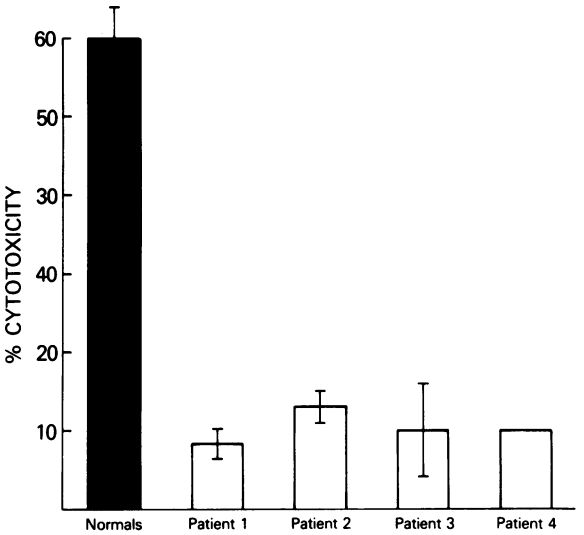

FIGURE 3 ADCC activity of normal and CGD neutrophils against rabbit IgG anti-HRBC-coated HRBC targets. At 100:1 E:T ratios, CGD neutrophils mediated significantly less ADCC than did normal neutrophils. Normal results were compiled from 11 separate experiments with different subjects. CGD patients 1–3 were studied on three separate occasions, whereas patient 4 was studied once.

cells in suspension in which it is difficult to determine the relative contribution of intracellular and extracellular killing. In an attempt to distinguish between these two mechanisms, monolayers of HRBC targets were

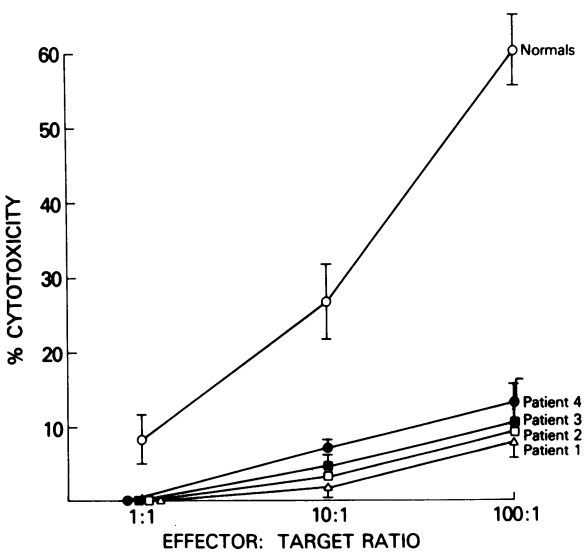

FIGURE 4 ADCC activity of normal and CGD neutrophils against rabbit IgG anti-HRBC-coated targets in suspension at 100:1, 10:1, and 1:1 E:T ratios. CGD neutrophils had markedly diminished ADCC activity at all ratios when compared to normals. Normal results were compiled from 11 separate experiments with different subjects. CGD patients 1–3 were studied on three separate occasions, whereas patient 4 was studied once.

employed. Using this system, effector cells can attach to but not ingest antibody-coated HRBC targets. As shown in Fig. 5, effector cells (lymphocytes, monocytes, and neutrophils) from four CGD patients were capable of mediating target cell lysis as measured by ^{51}Cr release comparable to effector cells from four normal donors ($P > 0.2$ for all).

Composition of effector cell populations. Because abnormalities of cytotoxic capabilities of CGD mononuclear cell suspensions might be reflecting alterations in the composition of these cell suspensions as compared to those of normal subjects, we compared them to those from normal subjects (Table I). Mononuclear cell suspensions from CGD patients were not significantly different with regard to the percentage of monocytes, lymphocytes, FcR^+ cells, B cells, or T cells when compared to normals. Similarly, neutrophil suspensions from these patients were comprised to relative numbers of total neutrophils and FcR^+ neutrophils comparable to normal neutrophil suspensions (Table I). Because phagocytic cell ADCC against antibody-coated HRBC may represent a postphagocytic event, we demonstrated that CGD monocytes and neutrophils were able to ingest 7S EA comparably to normals (Table II).

Effects of in vitro colchicine on ADCC activity.

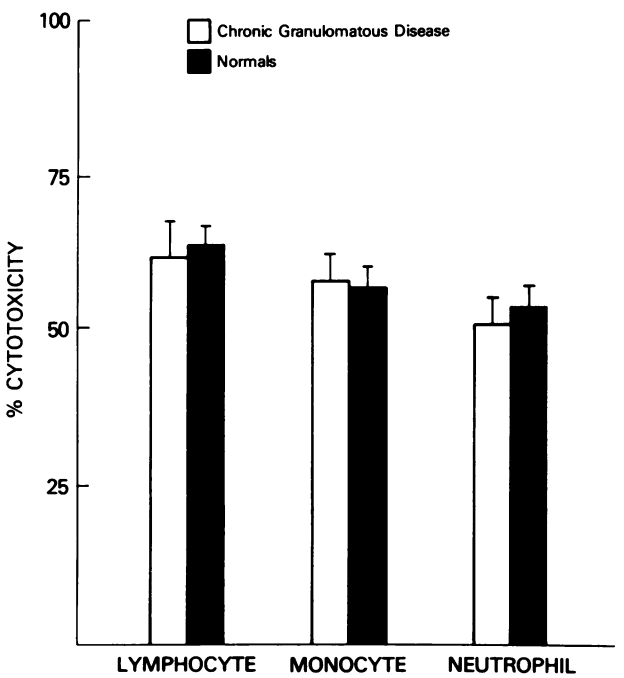

FIGURE 5 ADCC activity of normal and CGD effector cells against TNP-modified HRBC targets in monolayer with a ^{51}Cr-release assay. CGD effector cells-mediated ADCC comparably to normal effector cells. Data represent the mean of four separate experiments with four different CGD patients and four different normal subjects.

The above observations indicated that CGD effector cells could kill antibody-coated HRBC normally in an extracellular assay system (i.e., targets in monolayer) but could not kill these same targets normally in an intracellular assay (i.e., targets in suspension). Therefore, we next attempted to determine if by blocking phagocytosis of target cells by CGD cells in suspension culture, we could induce these effector cells to kill antibody-coated HRBC targets extracellularly. The addition of 1 mM–0.1 μM colchicine to normal or CGD mononuclear cell or neutrophil suspensions did not affect normal binding of 7S EA but did inhibit phagocytosis (Table III). As shown in Fig. 6A, 0.1 mM–0.1 μM colchicine had little effect on normal mononuclear cell-mediated ADCC against antibody-coated HRBC targets, but markedly enhanced the ADCC activity of mononuclear cells from CGD patient 2. The greatest increase in this lysis was demonstrated with 0.1 mM colchicine. 0.1 mM–0.1 μM colchicine did not augment the killing of antibody-coated HRBC targets in suspension mediated by monocyte-depleted lymphoid cells from either a normal subject or from patient 2 (data not shown). Presumably this killing is mediated by nonphagocytic, FcR^+ lymphocytes and occurs extracellularly and is not affected by colchicine.

Additionally, 10–0.1 μM colchicine did not affect normal neutrophil-mediated ADCC against HRBC targets but greatly enhanced the ADCC capabilities of neutrophils from patient 2 (Fig. 6B). 1mM–0.1 μM colchicine did not increase the spontaneous release of ^{51}Cr-labeled HRBC.

When 1 μM colchicine was added to TNP-modified HRBC targets in monolayer, no significant enhancement of ADCC was observed with neutrophils from either patient 2 or a normal subject (Fig. 7). However, using TNP-modified HRBC targets in suspension, 1 μM colchicine markedly augmented the neutrophil-mediated ADCC of patient 2, but resulted in only a slight increase in the ADCC activity of normal neutrophils. As described above, 1 μM colchicine alone did not increase the spontaneous release of ^{51}Cr from targets in monolayer.

ADCC activity against lymphoid targets in suspension. With use of autologous lymphoid targets, we have shown that ADCC activity can be mediated by lymphocytes and neutrophils, but not by monocytes, and that phagocytosis of these targets does not occur.[3] As an adjunct to the above studies, we determined that mononuclear cells (Fig. 8) and neutrophils (Fig. 9) from CGD patients killed autologous antibody-coated lymphoid cells normally. These findings remained the same regardless of the E:T ratio, dilution of antibody, or culture interval employed (data not shown).

[3] Katz, P., and A. S. Fauci. Manuscript in preparation.

TABLE I

Composition of Mononuclear and Neutrophil Effector Cell Populations in Normal Subjects and CGD Patients

	Normals (n = 11)	Patient No. 1*	2*	3*	4‡
Mononuclear cell suspensions					
% Monocytes	18±2	22±6§	15±4§	21±8§	20‖
% Lymphocytes	82±8	78±9§	85±10§	79±11§	80‖
% FcR^+ cells	31±4	25±6§	30±8§	28±10§	26‖
% B lymphocytes	11±5	13±3§	9±3§	14±5§	13‖
% T lymphocytes	61±8	56±5§	58±4§	68±7§	62‖
Neutrophil suspensions					
% Neutrophils	>95	>95‖	>95‖	>95‖	98‖
% FcR^+ cells	77±3	70±8§	83±6§	81±5§	85‖

* Mean±SEM of three separate experiments.
‡ Results of one experiment.
§ $P > 0.2$ compared to normals.
‖ Within range of normals.

DISCUSSION

The present study has clearly demonstrated that the ADCC activity of human phagocytic cells against antibody-coated human erythrocyte targets in suspension is predominantly an intracellular event that is dependent upon a normal postphagocytic respiratory burst. Normal phagocytes, the predominant effector cells in classic ADCC assays against HRBC targets in suspension (2), could rapidly bind, ingest, and lyse antibody-coated HRBC. CGD phagocytic cells, however, were capable of normal binding and ingestion of these targets but were unable to effect intracellular lysis. Because CGD phagocytes lack the burst of oxidative metabolism that normally occurs during phagocytosis (3–5), this would suggest that normal intracellular lysis of erythroid targets is dependent upon this activity.

Because of the findings of abnormal postphagocytic CGD monocyte and neutrophil-mediated ADCC against target cells in suspension, we investigated CGD effector cell capabilities against HRBC targets in monolayer. With this system, antibody-coated HRBC are bound by antibody to plastic surfaces. When exposed to these targets, FcR^+ mononuclear cells and neutrophils bind and kill these targets in the absence of phagocytosis.[2] By using ^{51}Cr-labeled HRBC targets in monolayer, we have demonstrated that CGD lymphocytes, monocytes, and neutrophils are capable of inducing ^{51}Cr release comparable to that observed when normal effector cells are employed. Thus, CGD effector cells, although incapable of cytotoxic activity against antibody-coated HRBC targets in suspension, can kill these targets normally when they are in monolayer.

TABLE II

Phagocytosis of 7S EA by Monocytes and Neutrophils from Normal Subjects and CGD Patients

	Normals (n = 11)	Patient No. 1*	2*	3*	4‡
% Monocytes with phagocytosed 7S EA	86±2	90±4§	82±7§	93±6§	84‖
% Neutrophils with phagocytosed 7S EA	39±3	42±5§	44±8§	37±4§	41‖

* Mean±SEM of three separate experiments.
‡ Results of one experiment.
§ $P > 0.1$ when compared to normals.
‖ Within range of normals.

TABLE III
Effect of In Vitro Colchicine on Phagocytosis of 7S EA by Normal and CGD Phagocytic Cells

	Concentration of colchicine					
	0	0.10 μM	1 μM	10 μM	0.1 mM	1 mM
% Monocytes with phagocytosed 7S EA						
Normal	85	5	0	0	0	0
Patient 2	83	8	0	0	0	0
% Neutrophils with phagocytosed 7S EA						
Normal	39	2	0	0	0	0
Patient 2	43	1	0	0	0	0

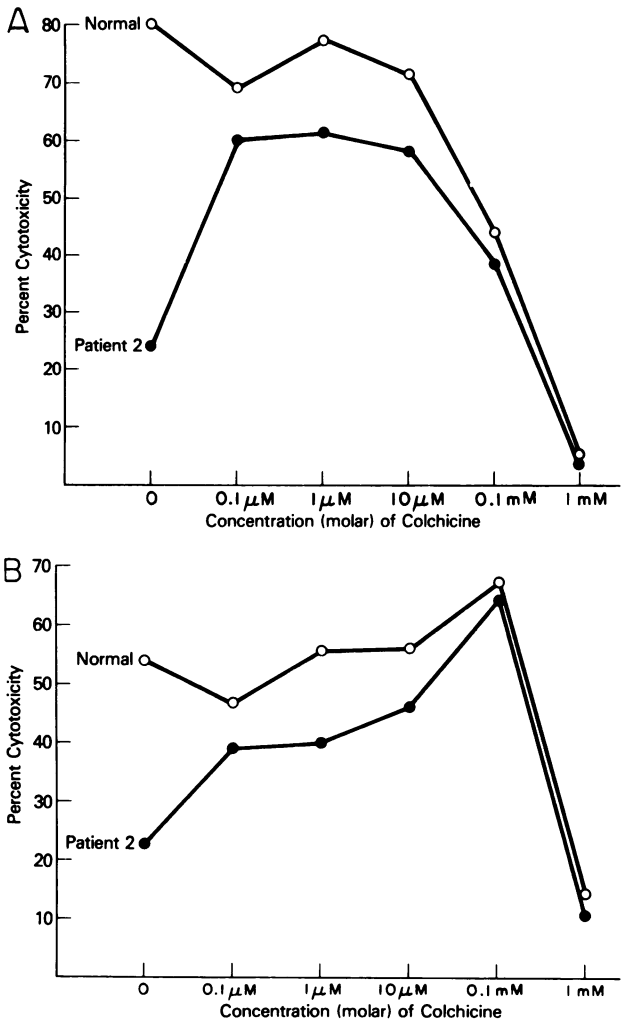

FIGURE 6 (A) Effect of in vitro colchicine on the ADCC activity of normal and CGD mononuclear cells against antibody-coated HRBC targets in suspension. In vitro 0.1 mM–0.1 μM colchicine increased the ADCC activity of mononuclear cells from CGD patient 2, while having little effect on the ADCC activity of mononuclear cells from a normal subject. (B) Effect of in vitro colchicine on the ADCC activity of normal and CGD neutrophils against antibody-coated HRBC targets in suspension. In vitro 10–0.1 μM colchicine increased the ADCC activity of neutrophils from CGD patient 2 while exerting little effect on the ADCC of neutrophils from a normal subject.

The addition of colchicine to suspension cultures in concentration that prevented phagocytosis but not binding of antibody-coated target cells (18–20) normalized the defective ADCC activity of CGD effector cells. Thus, by preventing phagocytosis and by permitting target cell lysis by extracellular means, CGD effector cells were able to kill normally.

The mechanism of inhibition of phagocytosis is unclear but may well involve the inhibition of microtubular assembly with an inhibition of the subsequent reorganization of membrane lipids and proteins necessary for particle ingestion (18, 19). Although some studies have not observed inhibition of phagocytosis by colchicine, this may be secondary to the use of different particles for ingestion or different assay systems. However, in our system, colchicine consistently inhibited the phagocytosis of antibody-coated erythrocytes.

Recently, Fleer et al. (20) reported colchicine-induced inhibition of lysis of sensitized human erythrocyte targets in suspension. However, these investigators used only 1 mM colchicine, a concentration that we likewise found to inhibit ADCC. At lower concentrations of this agent that still inhibited phagocytosis, we observed increased killing by CGD effector cells. Thus extracellular killing of antibody-coated target cells in ADCC appears to occur via mechanisms different from those of intracellular killing, and indeed the dichotomy is clearly exemplified in the CGD patients because killing is normal in the former assay and markedly depressed in the latter. Likewise through the use of antibody-coated autologous lymphoid targets in suspension culture, we have shown that CGD effector cells have normal ADCC activity against this

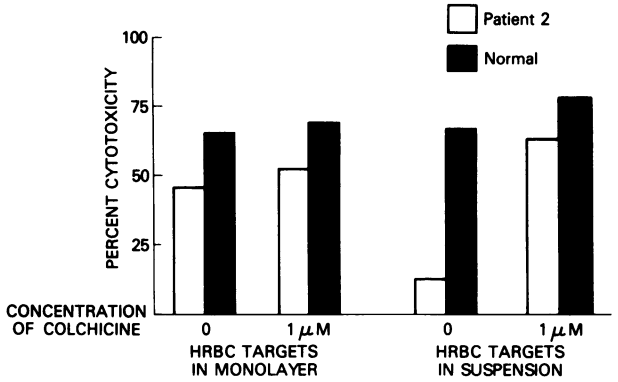

FIGURE 7 Effect of in vitro 1 μM colchicine on the ADCC activity of normal neutrophils and neutrophils from CGD patient 2 against TNP-modified HRBC targets in suspension and in monolayer. Colchicine increased CGD neutrophil-mediated ADCC against targets in suspension without affecting normal neutrophil-mediated ADCC. Colchicine did not affect ADCC activity against targets in monolayer.

target, which is normally lysed extracellularly. Because the abnormality in CGD phagocytic cells is a defect in the normal postphagocytic respiratory burst, and because CGD phagocytic cells manifest abnormal ADCC in suspension that is an intracellular killing with normal ADCC in the monolayer assay that is an extracellular killing, it appears then that intracellular postphagocytic ADCC is dependent upon a normal postphagocytic respiratory burst with the generation of H_2O_2 and O_2^-.

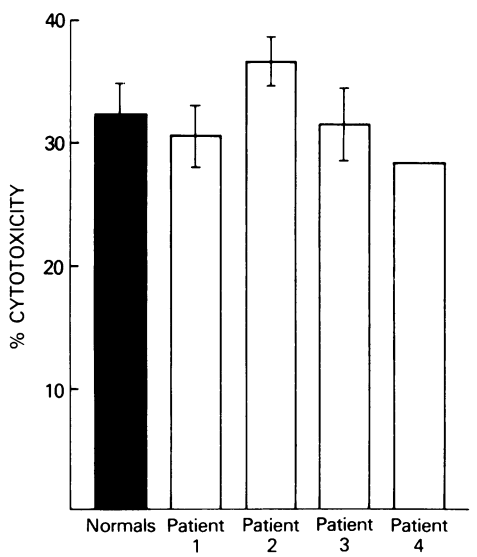

FIGURE 8 ADCC activity of normal and CGD mononuclear cells against autologous rabbit IgG ALS-coated lymphoid cells in suspension. A 100:1 E:T ratios, CGD effector cells displayed normal ADCC activity. Normal results were compiled from 11 separate experiments with different subjects. CGD patients 1–3 were studied on three separate occasions, whereas patient 4 was studied once.

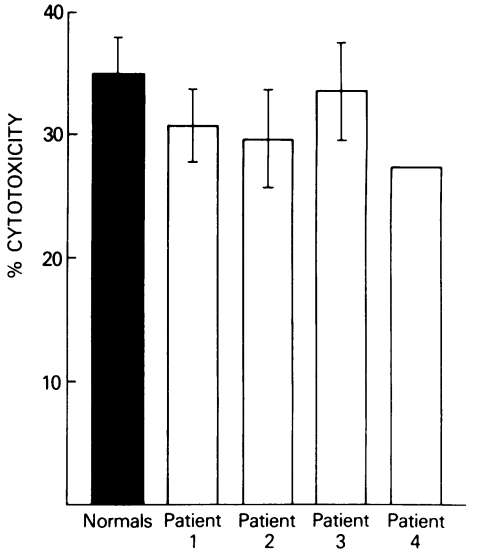

FIGURE 9 ADCC activity of normal and CGD neutrophils against autologous rabbit IgG ALS-coated lymphoid cells. At 100:1 E:T ratios, CGD neutrophils mediated normal ADCC activity. Normal results were compiled from 11 separate experiments with different subjects. CGD patients 1–3 were studied on three separate occasions, whereas patient 4 was studied once.

Conversely, extracellular ADCC, which is mediated normally by CGD effector cells that lack the ability to generate these products, is in all likelihood dependent upon a different metabolic pathway(s).

To date, the exact mechanisms of ADCC activity have been controversial. Clark and Klebanoff (21) using neutrophil effector cells against antibody-coated mammalian tumor cells demonstrated the dependence of this activity on a burst of oxidative metabolism, glycolysis, divalent cations, and microtubular function. CGD effector cells were found by these investigators to have depressed ADCC activity against these targets, thus suggesting the requirement for a metabolic burst. This study as well as that of Fleer et al. (20) suggested that target cell lysis might be dependent upon the extracellular release of the contents of neutrophil and monocyte granules.

Additional studies on the mechanism of ADCC have indicated that this activity was dependent on the maintenance of certain optimal levels of total energy production (22). Thus, the inhibition of both aerobic and anaerobic energy production totally prevented the lysis of antibody-coated cells of a lymphoblastoid cell line (22). Studies investigating the mechanism of nonantibody-dependent cytotoxicity have reported conflicting results. MacDonald and Koch (23) have determined that T cell-mediated cytolysis was energy dependent and could be mediated by either glycolytic or oxidative pathways. By using mice macrophages as effector cells, Sorell and co-workers (24) determined that the lysis of

allogeneic virus-transformed fibroblasts was not dependent upon the release of either H_2O_2 or O_2^-. Conversely, however, three separate studies have indicated that extracellular cytolysis can be mediated by H_2O_2 (25–27). With effector cells from CGD patients as investigative probes, these studies have indicated that intracellular ADCC is largely a postphagocytic event and as such is dependent upon an intact postphagocytic burst of oxidative metabolism.

Thus, standard ADCC against HRBC targets in suspension may primarily reflect intracellular target lysis. Extracellular target cell lysis, however, appears to be independent of the generation of H_2O_2 and/or O_2^- and additionally may be independent of the action of lysosomal granules. Hopefully, additional studies in this area will further clarify the mechanisms of ADCC.

REFERENCES

1. Pearson, G. R. 1978. In vitro and in vivo investigations on antibody-dependent cellular cytotoxicity. *In* Contemporary Topics in Microbiology and Immunology. W. Arber, W. Henle, P. H. Hofschneider, J. H. Humphrey, J. Klein, P. Koldovsy, H. Koprowski, O. Maaloe, F. Melchers, R. Rott, H. G. Schweiger, L. Syrucek, and P. K. Vogt, editors. Springer-Verlag, Berlin. **80:** 65–96.
2. MacDonald, H. R., G. D. Bonnard, B. Sordat, and S. A. Zawodnik. 1975. Antibody-dependent cell-mediated cytotoxicity: heterogeneity of effector cells in human peripheral blood. *Scand. J. Immunol.* **4:** 487–497.
3. Holmes, B., and A. R. Page. 1966. Metabolic abnormalities of leukocytes from children with CGD. *J. Cell Biol.* **31:** 48a–49a.
4. Baehner, R. L., and D. G. Nathan. 1967. Leukocyte oxidase: defective activity in chronic granulomatous disease. *Science (Wash. D. C.).* **155:** 835–836.
5. Hohn, D. C., and R. I. Lehrer. 1975. NADPH oxidase deficiency in X-linked chronic granulomatous disease. *J. Clin. Invest.* **55:** 707–713.
6. MacPherson, B. R. 1977. The clinical and laboratory diagnosis of chronic granulomatous disease of childhood. *CRC Crit. Rev. Clin. Lab. Sci.* **8:** 81–103.
7. Bridges, R. A., H. Berendes, and R. A. Good. 1959. A fatal granulomatous disease of childhood: the clinical, pathological, and laboratory features of a new syndrome. *Am. J. Dis. Child.* **97:** 387–408.
8. Park, B. H., S. M. Fikrig, and E. M. Smithwick. 1968. Infection and nitroblue tetrazolium reduction by neutrophils: a diagnostic aid. *Lancet.* **II:** 532–534.
9. Klempner, M. S., C. A. Dinarello, W. R. Henderson, and J. I. Gallin. 1979. Stimulation of neutrophil oxygen-dependent metabolism by human leukocytic pyrogen. *J. Clin. Invest.* **64:** 996–1002.
10. Weening, R. S., R. Wever, and D. Roos. 1975. The production of superoxide radicals by phagocytosing leukocytes. *J. Lab. Clin. Med.* **85:** 245–252.
11. Boyum, A. 1968. Separation of leucocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest.* **21**(Suppl): 77–89.
12. Parish, C. R., and J. A. Hayward. 1974. Separation of Fc receptors, C'3 receptor and surface immunoglobulin-bearing lymphocytes. *Proc. R. Soc. Lond. B Biol. Sci.* **187:** 65–81.
13. Parrillo, J. E., and A. S. Fauci. 1978. Mechanisms of corticosteroid action on lymphocyte subpopulations. IV. Differential effects of dexamethasone administration on subpopulations of effector cells mediating cellular cytotoxicity in man. *Clin. Exp. Immunol.* **31:** 116–125.
14. Boyum, A. 1976. Isolation of lymphocytes, granulocytes and macrophages. *Scand. J. Immunol.* **5:** 9–15.
15. Kedar, E., M. Ortiz DeLandazuri, and J. L. Fahey. 1974. Comparative studies of immunoglobulin receptors and antibody-dependent cell cytotoxicity (ADCC) in rat lymphoid organs. *J. Immunol.* **112:** 37–46.
16. Klempner, M. S., and J. I. Gallin. 1978. Separation and functional characterization of human neutrophil subpopulations. *Blood.* **51:** 659–669.
17. Nelson, D. L., D. G. Poplack, B. J. Holiman, and P. A. Henkart. 1979. ADCC against human erythrocyte target cells: role of the anti-target cell antibodies in determining lymphocyte killer activity. *Clin Exp. Immunol.* **35:** 447–453.
18. Stossel, T. P., R. J. Mason, J. Hartwig, M. Vaughan. 1972. Quantitative studies of phagocytosis by polymorphonuclear leukocytes: use of emulsions to measure the initial rate of phagocytosis. *J. Clin. Invest.* **51:** 615–624.
19. Lehrer, R. I. 1973. Effects of colchicine and chloramphenicol on the oxidative metabolism and phagocytic activity of human neutrophils. *J. Infect. Dis.* **127:** 40–48.
20. Fleer, A., M. L. J. Van Schark, A. E. G. Kr. Von Dem Boine, and C. P. Engelfreit. 1978. Destruction of sensitized erythrocytes by human monocytes in vitro: effects of cytochalasin B, hydrocortisone and colchicine. *Scand. J. Immunol.* **8:** 515–524.
21. Clark, R. A., and S. A. Klebanoff. 1977. Studies on the mechanism of antibody dependent polymorphonuclear leukocyte-mediated cytotoxicity. *J. Immunol.* **119:** 1413–1418.
22. Trinchieri, G., and M. DeMarchi. 1975. Antibody-dependent cell-mediated cytotoxicity in humans. II. Energy requirements. *J. Immunol.* **115:** 256–260.
23. MacDonald, H. R., and C. J. Koch. 1977. Energy metabolism and T-cell-mediated cytolysis. I. Synergism between inhibitors of respiration and glycolysis. *J. Exp. Med.* **146:** 698–709.
24. Sorrell, T. C., R. I. Lehrer, and M. J. Cline. 1978. Mechanism of nonspecific macrophage-mediated cytotoxicity: evidence for lack of dependence upon oxygen. *J. Immunol.* **120:** 347–352.
25. Edelson, P. J., and Z. A. Cohn. 1973. Peroxidase-mediated mammalian cell cytotoxicity. *J. Exp. Med.* **138:** 318–323.
26. Clark, R. A., S. J. Klebanoff, A. B. Einstein, and A. Fefer. 1975. Peroxidase-H_2O_2-halide system; cytotoxic effect on mammalian tumor cells. *Blood.* **45:** 161–170.
27. Nathan, C. F., S. C. Silverstein, L. H. Brukner, and Z. A. Cohn. 1979. Extracellular cytolysis by activated macrophages and granulocytes. I. Hydrogen peroxide as a mediator of cytotoxicity. *J. Exp. Med.* **149:** 100–113.