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Cytotoxicity of Human Peripheral Blood and Colostral Leukocytes Against *Shigella* Species

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We examined the ability of human peripheral blood leukocytes to kill strains of *Shigella sonnei* and *Shigella flexneri* by using a modified bactericidal assay. Antibody-dependent cellular cytotoxicity (ADCC) was demonstrated in the presence of specific rabbit immune serum directed against *S. sonnei*. With peripheral blood leukocytes from adults, ADCC was found only in the mononuclear cell and purified lymphocyte populations. Monocyte-macrophages and polymorphonuclear leukocytes were unable to demonstrate ADCC. Lymphocyte ADCC, which was not affected by the addition of phenylbutazone (an inhibitor of phagocytosis), was mediated by a non-T, Fc receptor-positive, HNK-1$^-$ cell. ADCC (using antiserum directed against virulent *S. sonnei*) was demonstrated against virulent *S. sonnei* but not against avirulent *S. sonnei* or virulent *S. flexneri*. In contrast to leukocytes from adults, both mononuclear and polymorphonuclear cells from neonatal cord blood and from a patient with chronic granulomatous disease mediated anti-*Shigella* ADCC. Breast milk leukocytes (BMLs) collected 1 to 3 days postpartum were used as effector cells against virulent *S. sonnei*. The entire BML population, BMLs which did not adhere to plastic and BMLs which passed through nylon wool columns mediated both natural killer cytotoxicity and ADCC. In paired experiments, natural killer cytotoxicity and ADCC were significantly lower (30 to 45% inhibition) but not ablated, when phenylbutazone was added to BMLs and nylon wool-purified BMLs ($P < 0.05$). These experiments suggest that colostral leukocytes mediated both extracellular and intracellular bacteriolysis in the presence and absence of specific antiserum. These mechanisms may be active in vivo in protection against shigellosis.

It has been demonstrated that leukocytes can, in the presence of specific antibody, cause extracellular lysis of a variety of target cells, such as tumor cells (3, 16, 24, 31, 35), virus-infected cells (16, 19, 22, 23), parasites (16, 30), fungi (7, 16, 33), and bacteria (13, 28, 29, 34). This antibody-dependent cellular cytotoxicity (ADCC) occurs in the absence of complement. Nonimmune effector cells, which function without restriction by the major histocompatability antigens, are able to cause extracellular lysis of antibody-coated target cells. ADCC is mediated by K cells and other effector cells. ADCC-mediated lysis is Fc receptor dependent (35). Natural killer cytotoxicity (NKC) is a second extracellular leukocyte killing mechanism. NKC occurs in the absence of antibody and complement. NKC, which is Fc receptor independent, is mediated by NK cells and other nonimmune effector cells. Although the antibacterial phagocytic capabilities of monocytes and polymorphonuclear cells are well characterized (2, 12, 25, 40, 41), less is known regarding the extracellular, nonphagocytic cytotoxicity mechanisms against bacteria.

Shigellosis is an important cause of morbidity and mortality worldwide (5, 6, 9, 42). Children between the ages of 1 and 10 years are most frequently infected (5, 6, 9). Neonatal shigellosis is not common, especially in breastfed infants (9, 32, 38). The immunity against *Shigella* spp. is unclear. During shigellosis, an intensive inflammatory response is manifested, as evidenced by the presence of fecal leukocytes (9, 15, 37). The role of these leukocytes in immunity is uncertain. Local gut-level immunity is believed to be important (4, 8, 9, 47). Since intraepithelial lymphocytes of the gut have been demonstrated to mediate NKC and ADCC in other systems (3, 34), we speculated an in vivo role for NKC and ADCC against *Shigella* spp. at the gut level, where cytotoxic effector cells and immunoglobulins are abundant. We studied the mechanisms of NKC and ADCC of human peripheral blood leukocytes (PBLs) and breast milk leukocytes (BMLs) in vitro against virulent and avirulent strains of *Shigella* spp.

MATERIALS AND METHODS

Bacterial strains. Virulent strains of *Shigella sonnei* (53GI) and *Shigella flexneri* (M4243) were kindly provided by Samuel Formal (Walter Reed Army Hospital, Washington, D.C.). Recent, virulent, patient isolates of *S. sonnei* (BI5V), BI18V, and UT2) and *S. flexneri* (AB43) were collected from patients with acute diarrhea in Guadalajara, Mexico. *Escherichia coli* ATCC 25922 was used in the phagocytosis assays. Phase I (smooth) colonies were cultured for 3 to 5 h at 37°C in 10 ml of Casamino Acids-yeast extract broth for use as the virulent target cells in the bactericidal assay (10). In some experiments, phase II (rough) colonies were chosen as avirulent target cells. Inocula were standardized to ca. 10^5 CFU/ml by optical density.

Antisera. Hyperimmune rabbit serum was raised against strain 53GI by administering three biweekly intramuscular injections of heat-killed bacteria (at a concentration of 10^8 CFU per rabbit) plus Freund adjuvant (Difco Laboratories, Detroit, Mich.). Complete Freund was used for the first injection, and incomplete Freund was used for the remaining two injections. Rabbits were exsanguinated 2 weeks after the final injection. All antisera were pooled and heat treated at 56°C to inactivate complement before use in the bactericidal assay. Normal human serum was used in the phagocytosis assays without heat inactivation.

PBL preparations. All protocols were reviewed and approved by the University of Texas Health Science Center

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Committee for the Protection of Human Subjects. Heparinized (10 U/ml of blood) peripheral blood was collected from healthy laboratory personnel. In some experiments, blood was collected from consenting women and their children. Blood was collected from healthy children as well as from a child with confirmed chronic granulomatous disease (CGD). The entire leukocyte population was collected after sedimentation in 10% (vol/vol) dextran (3%; Sigma Chemical Co., St. Louis, Mo.). The mononuclear cells or polymorphonuclear leukocytes were collected by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, N.J.) density gradient centrifugation at 300 × *g* for 45 min. After the mononuclear cells were washed with Hanks balanced saline solution (M. A. Bioproducts, Rockville, Md.), they were resuspended in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 20% heat-inactivated fetal calf serum plus 1% penicillin-streptomycin solution (GIBCO Laboratories). The mononuclear cells were then incubated in sterile petri dishes (20 by 100 mm; American Scientific Products, Houston, Tex.) for 18 h at 37°C under a 5% CO_2 humidified atmosphere. The majority of the monocyte-macrophage population adhered to the plastic dishes, and any contaminating granulocytes underwent autolysis during this incubation as previously described (48). In some experiments, the lymphocytes were passed over nylon wool columns, as previously described, to remove B cells and macrophages (18). The purified leukocytes were washed twice in Hanks balanced saline solution, resuspended in minimal essential medium (MEM; M. A. Bioproducts), counted with a hemacytometer, and diluted as required in MEM for use as effector cells in the bactericidal assay.

Cord blood leukocyte preparation. Umbilical cord blood was collected in heparinized syringes at the time of delivery and subjected to Ficoll-Hypaque density gradient centrifugation, as described above. Mononuclear and polymorphonuclear cells were washed, counted, and diluted as required in MEM for use as effector cells in the bactericidal assay without any further purification procedures.

BML collection and preparation. Colostral samples were collected from consenting, healthy mothers (1 to 3 days postpartum) with an Egnell breast pump (Egnell, Inc., Cary, Ill.). Samples were centrifuged at 500 × *g* to remove the lipid and aqueous material. The leukocytes were then washed twice with Hanks balanced saline solution (M. A. Bioproducts) and resuspended in RPMI 1640 medium (M. A. Bioproducts) plus 10% heat-inactivated fetal calf serum. In some experiments, the cells were placed in 25-cm^2 tissue culture flasks from which HeLa cell monolayers had been removed to enhance the ability of macrophages to adhere to the plastic. After a 2-h incubation at 37°C in air plus 5% CO_2, the nonadherent cell population was collected, washed in Hanks solution, and resuspended in MEM (M. A. Bioproducts) for use as effector cells in the bactericidal assay. In additional experiments, BMLs were passed through two nylon wool columns. The cells which passed through the columns were washed and resuspended in MEM.

E rosette assay. T cells were selected by their ability to form spontaneous rosettes with sheep erythrocytes (SRBC), as described previously (14, 39). Briefly, 1% SRBC (Flow Laboratories, Inc., McLean, Va.) in RPMI 1640 medium were treated with 20 U of neuraminidase (GIBCO Laboratories) per ml. Fetal calf serum was absorbed with 1% SRBC. Equal volumes of 1% SRBC, absorbed fetal calf serum, and purified lymphocytes (10^7 cells per ml) were layered over 10 ml of Ficoll-Hypaque. After a 30-min incubation at 4°C and centrifugation at 300 × *g* for 45 min, the nonrosetting cells were collected at the interface of the gradient. E-rosetting cells were collected from the pellet after lysis of the SRBC with distilled water. The nonrosetting cells and rosetting cells were washed and used as effector cells in the bactericidal assay.

EA rosette assay. Fc receptor-positive cells were selected by their ability to form rosettes with antibody-coated SRBC, as described previously (14, 39). Briefly, 1% SRBC were sensitized with rabbit anti-SRBC (immunoglobulin M; Cordis Laboratories, Miami, Fla.) according to the instructions of the manufacturer. Equal volumes of sensitized SRBC and purified lymphocytes were layered over 10 ml of Ficoll-Hypaque. After a 2-h incubation at 4°C and centrifugation at 300 × *g* for 45 min, the nonrosetting cells (Fc^-) were collected at the interface of the density gradient. EA rosetting cells (Fc^+) were collected in the pellet after lysis of the SRBC. Fc^+ and Fc^- cells were washed and used as effector cells in the bactericidal assay.

Complement-dependent cytotoxicity. In some experiments, effector cells were lysed before their use in the bactericidal assay with specific antibodies in the presence of complement. Effector cells (6×10^7 cells per ml) were preincubated at 4°C for 60 min with 60 μg of anti-Leu-7 (Becton Dickinson and Co., Paramus, N.J.), a monoclonal antibody directed against the cell surface marker of human NK cells (1). Low-toxicity rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) was added after centrifugation and incubated at 37°C for 60 min. Percent cytotoxicity was determined by trypan blue exclusion after centrifugation to remove complement.

Bactericidal assay. A modified bactericidal assay was used (41). Bacteria suspended in Casamino Acids-yeast extract broth were added to U-bottom microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) in 50-μl volumes to obtain a final effector (leukocyte)-to-target (bacteria) cell ratio (E:T) ranging from 30:1 to 100:1. Rabbit anti-*Shigella* serum or normal rabbit serum at a final dilution ranging from 1:10 to 1:1000 was added in a 100-μl volume. The microtiter plates were incubated at 37°C for 15 to 120 min. Most assays were performed with a 60-minute incubation period. Three serial 10-fold dilutions of the leukocyte and bacteria mixture were plated in duplicate onto Tergitol-7 agar. One percent triphenyltetrazolium chloride (Eastman Kodak Co., Rochester, N.Y.) was added to the agar to more easily visualize the colonies. All dilutions were performed in distilled water to lyse the leukocytes. In some experiments, the assay mixture was sonicated to disrupt leukocytes before preparation of the dilutions. The plates were incubated at 37°C for 18 h, and the colonies were counted. The NKC was calculated as follows: NKC = 100 − 100 (CFU of bacteria + leukocytes + normal serum/CFU of bacteria without leukocytes). The ADCC was calculated as follows: ADCC = 100 − 100 (CFU of bacteria + leukocytes + immune serum/CFU of bacteria + leukocytes). CFU is CFU per milliliter. Therefore, NKC represents the percentage of bacteria killed in the presence of leukocytes alone. ADCC represents the additional percentage of bacteria killed in the presence of leukocytes and immune serum.

Phagocytic assay. A modified assay for phagocytosis of *E. coli* was used (26, 27, 43). Bacteria suspended in Casamino Acids-yeast extract broth were added to sterile microtiter plates in 50-μl volumes. Fresh human serum was added in 100-μl volumes at a final dilution of 1:10. After a 60-min incubation at 37°C, 50 μl of polymorphonuclear leukocytes was added to equal E:T's of 60:1. The plates were incubated for an additional 60 min. Three serial 10-fold dilutions (in distilled water) of the leukocyte-bacteria suspension were plated onto Tergitol-7 agar containing 1% triphenyltetrazo-

lium chloride. The plates were incubated overnight at 37°C before the colonies were counted. A phagocytic antibacterial index (ABI) was calculated by the following equation: ABI = 100 − 100 (CFU of bacteria + leukocytes + serum/CFU bacteria alone), where CFU is CFU per milliliter.

Inhibition by phenylbutazone. In some experiments effector cells and target cells were suspended in medium containing 2 mg of phenylbutazone (Sigma Chemical Co., St. Louis, Mo.) per ml to inhibit intracellular killing as a result of phagocytosis (26, 27, 43).

Anti-HSV-1 cytotoxicity assay. A standard 18-h chromium release assay was performed as previously described (19, 22, 23). Chang liver cells were infected with herpes simplex virus type 1 (HSV-1; HE strain) 18 h before use, labeled with radioactive sodium chromate (^{51}Cr), and suspended to a final concentration of 10^5 cells per ml in MEM plus 10% fetal calf serum. The cytotoxicity assay was performed in 96-well U-bottom microtiter plates with 100 μl of effector cells, 50 μl of target cells, and 50 μl of nonimmune or immune human serum (with an anti-HSV neutralizing titer of 1:32 and ADCC titer of 10^{-5}) and incubated for 18 h at 37°C in 5% CO_2.

To determine the amount of ^{51}Cr released from the target cells, 100 μl was aspirated from the top of each well without disturbing the cell button. To each well, 100 μl of 1 M NaOH was added, and the total volume was aspirated into a separate container. All samples were counted in a Beckman Biogamma gamma counter for 1 min. Chromium release was calculated by the following formula: $\%^{51}Cr$ released = $2A/A + B$, where A equals counts per minute in the top 100 μl, and B equals counts per minute in the bottom 100 μl to which NaOH was added. NKC was calculated as follows: NKC = ($\%^{51}Cr$ release of target cells and effector cells − $\%^{51}Cr$ release of target cells alone/100 − $\%^{51}Cr$ release of targets cells alone) × 100. ADCC was calculated as follows: ADCC = ($\%^{51}Cr$ release from target cells, effector cells, and immune serum − $\%^{51}Cr$ release of target cells and effector cells alone/100 − $\%^{51}Cr$ release of target cells alone) × 100.

Statistics. Statistical analysis was performed by analysis of variance, two-tailed Student's t test, and least-significant difference test.

RESULTS

PBL ADCC. NKC was not demonstrated with PBL effector cells. ADCC against strain 53GI of the classes of leukocytes was examined with a 1:10 dilution of antiserum in a 60-min bactericidal assay (Table 1). Mononuclear cells at an E:T of 100:1 significantly mediated ADCC ($P < 0.02$). A mean ADCC index of 36.5 was shown in 12 experiments. Purified lymphocytes also demonstrated ADCC at an E:T of 100:1 ($P < 0.05$). In 25 experiments, utilizing nine different donors, the mean ADCC was 46.4. Macrophages, obtainable only at an E:T of 50:1, did not mediate ADCC ($P > 0.4$). Similarly, polymorphonuclear cells at an E:T of 100:1 did not mediate ADCC ($P > 0.4$).

Lymphocyte ADCC. The first series of experiments with purified lymphocytes from peripheral blood was designed to determine the optimum concentration of antiserum necessary to mediate ADCC against strain 53GI. In three experiments with lymphocytes at an E:T of 100:1 in a 60-min bactericidal assay, three concentrations of antiserum were used (Fig. 1). Maximum ADCC was demonstrated with a final serum dilution of 1:10 ($P < 0.01$). No ADCC was demonstrated with normal (preimmune) rabbit serum, fetal calf serum, heat-killed leukocytes, or supernatants of lymphocytes and bacteria (data not shown). All subsequent assays were performed with a 1:10 final dilution of serum.

TABLE 1. PBL antibody-dependent cellular cytotoxicity against *S. sonnei* 53GI

Effector cells	n^a	E:T	Mean ± SE		
			PBLs alone[b]	PBLs + antibody[c]	ADCC[d]
Mononuclear cells	12	100:1	90.4 ± 14.2	51.8 ± 7.0	36.5 ± 7.5
Lymphocytes	25	100:1	118.8 ± 9.3	66.1 ± 8.3	46.4 ± 4.9
Macrophages	4	50:1	120.5 ± 16.1	121.0 ± 10.0	2.0 ± 2.0
Polymorphonuclear cells	9	100:1	102.3 ± 21.3	92.7 ± 25.2	7.8 ± 3.4

[a] Number of experiments.
[b] CFU (×10^3) per milliliter in the presence of PBLs alone.
[c] CFU (×10^3) per milliliter in the presence of PBLs plus immune serum directed against the homologous strain.
[d] Calculated ADCC. No antibody-independent cellular cytotoxicity was noted in any of the experiments. $P < 0.001$ by one-way analysis of variance when the ADCC values of all four effector cell populations were compared; $P < 0.02$ and $P < 0.001$ when the colony counts, using mononuclear cells and lymphocytes, respectively, were compared by a t test in the presence and absence of antibody; $P > 0.4$ when the colony counts, using macrophages and polymorphonuclear cells, were compared by a t test in the presence and absence of antibody.

ADCC of purified peripheral blood lymphocytes against strain 53GI was studied at four E:T's (Fig. 2). It was demonstrated that ADCC increased as the E:T increased. No ADCC was seen with an E:T of 3:1. The greatest increases in ADCC were seen with an E:T of 100:1. No increase in ADCC was seen with an E:T of 200:1. Subsequent assays were generally performed with an E:T of 100:1. Passage of lymphocytes over nylon wool columns to remove B cells and macrophages or sonication of the assay mixture to prevent possible leukocyte-mediated bacterial clumping did not alter lymphocyte ADCC (data not shown).

The kinetics of peripheral blood lymphocyte ADCC against strain 53GI were examined in two experiments with an E:T of 100:1 (Fig. 3). Little or no ADCC occurred after 15 and 30 min of incubation. A sharp increase in ADCC was seen after 60 min ($P < 0.05$). No further increase in ADCC was seen after 120 min. Subsequent experiments were performed with a 60-min incubation period.

Specificity of antiserum. The levels of peripheral blood lymphocyte ADCC against four species of *S. sonnei* and two species of *S. flexneri* were examined (Table 2). ADCC was demonstrated against the virulent forms (phase I) of all strains of *S. sonnei*. ADCC against the avirulent forms (phase II) was significantly lower ($P < 0.001$). No ADCC was observed against either strains of *S. flexneri* ($P < 0.001$). This series of experiments demonstrates the specificity of the antibody for virulent *S. sonnei*.

Effector cells of lymphocyte ADCC. To characterize the peripheral blood lymphocyte effector cell, lymphocytes were separated into E-rosetting (T) and non-E-rosetting (non-T) subpopulations. Likewise, lymphocytes were separated into EA-rosetting (Fc^+) and non-EA-rosetting (Fc^-) subpopulations. In a series of three experiments, non-T cells (<20% of lymphocytes) were demonstrated as the effectors of anti-*Shigella* ADCC (Table 3). T cells failed to mediate ADCC. Similarly, Fc receptor-positive cells (<20% of lymphocytes) mediated ADCC. Cells which lacked Fc receptors were not demonstrated as effector cells of anti-*Shigella* ADCC. Unseparated lymphocytes mediated ADCC in all cases. To determine whether HNK-1^+ lymphocytes were the effector cells of anti-*Shigella* ADCC, complement-dependent cytotoxicity assays were performed to selectively kill HNK-1^+ cells. A monoclonal antibody (α Leu-7) directed against the

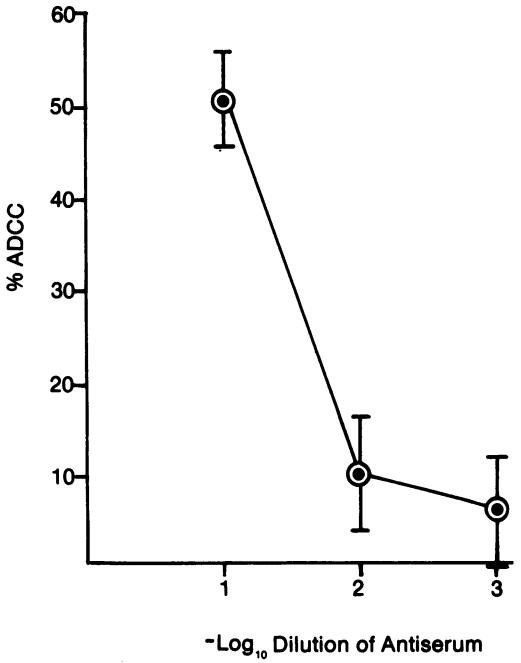

FIG. 1. Peripheral blood lymphocyte antibody-dependent cellular cytotoxicity against *S. sonnei* 53GI at various concentrations of antiserum in a 60-min bactericidal assay with an E:T of 100:1. The mean and standard error of three experiments are shown.

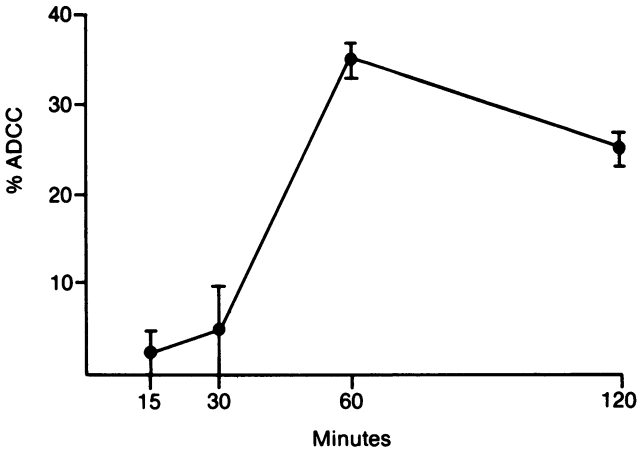

FIG. 3. Kinetics of peripheral blood lymphocyte antibody-dependent cellular cytotoxicity against *S. sonnei* 53GI in a 60-min bactericidal assay with an E:T of 100:1 and a final serum dilution of 1:10.

HNK-1 antigen of NK cells was used. An anti-HSV-1 assay was used as a control system in which one of the effector cells is HNK-1^+ (Table 4). Anti-*Shigella* ADCC was not inhibited by depletion of the HNK-1^+ subpopulation of lymphocyte effector cells (nearly 20% of the cells were killed, as determined by trypan blue exclusion). Anti-HSV NKC and ADCC, however, were decreased by treatment of effector cells with α Leu-7 plus complement.

Mechanism of peripheral blood lymphocyte ADCC. To confirm that the anti-*Shigella* cytotoxicity that was demonstrated was the result of an extracellular killing mechanism, phenylbutazone (2 mg/ml), which is an inhibitor of phagocytosis (26, 27, 43), was added to the medium for the bacteria, leukocytes, and serum. A phagocytic assay, with *E. coli* and polymorphonuclear cells, was used as a positive control (Table 5). Lymphocyte anti-*Shigella* ADCC was unaffected by the presence of phenylbutazone during the assay. Phagocytosis of *E. coli* by polymorphonuclear leukocytes, however, was markedly inhibited by the presence of phenylbutazone.

Leukocytes from a patient with CGD were used as effector cells to clarify whether intracellular killing via a metabolic burst was necessary for anti-*Shigella* ADCC. Mononuclear cells from this patient mediated ADCC, a result not different from that with normal adult controls (Table 6). However, polymorphonuclear cells from this patient mediated ADCC (in two experiments), to a level higher than that of adult polymorphonuclear cell ADCC.

Ontogeny of PBL ADCC. To determine the age-related development of anti-*Shigella* ADCC, mononuclear and poly-

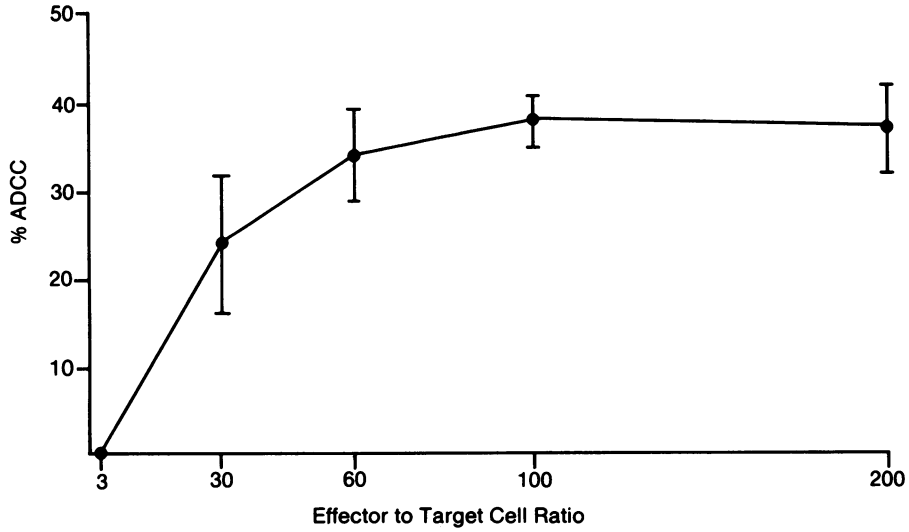

FIG. 2. Peripheral blood lymphocyte antibody-dependent cellular cytotoxicity against *S. sonnei* 53GI at various E:T's in a 60-min bactericidal assay with a final serum dilution of 1:10. The mean and standard error of five experiments are shown.

TABLE 2. Heterologous lymphocyte antibody-dependent cellular cytotoxicity against *Shigella* spp.

Target strain	Virulent		Avirulent	
	n^a	ADCC[b] (mean ± SE)	n^a	ADCC[b] (mean ± SE)
S. sonnei				
53GI	9	42.3 ± 6.4	ND[c]	ND
BI5V	4	29.0 ± 5.0	2	23.0 ± 10.1
UT2	4	32.5 ± 5.3	2	4.0 ± 4.0
BI18V	1	49.0	2	18.5 ± 8.6
S. flexneri				
M4243	3	3.3 ± 3.3	ND	ND
AB43	1	0	ND	ND

[a] Number of experiments.
[b] Calculated ADCC of experiments performed with an E:T of 100:1 and immune serum directed against strain 53GI. $P < 0.0001$ by analysis of variance when the ADCC of virulent *S. sonnei*, avirulent *S. sonnei*, and virulent *S. flexneri* were compared; $P < 0.05$ when the ADCC of virulent *S. sonnei* was compared to the ADCC of avirulent *S. sonnei* and virulent *S. flexneri* by the least-significant difference test.
[c] Experiments not performed.

morphonuclear cells from adults, young children, and neonates were used at an E:T of 100:1 in a 60-min bactericidal assay against strain 53GI (Table 6). Mononuclear cells from all three populations mediated ADCC. Mononuclear cells from neonatal cord blood mediated higher ($P < 0.05$) ADCC than did cells from adults. As shown previously, polymorphonuclear cells from adults did not mediate ADCC. However, polymorphonuclear cells from neonates, like those from the CGD patients, did mediate ADCC. The ADCC of polymorphonuclear cells from neonates was significantly higher than that from the adults ($P < 0.05$).

BML NKC and ADCC. A series of three experiments was performed with the entire BML population at E:T's ranging from 3:1 to 100:1 and a constant serum dilution of 1:10 (Fig. 4). Unlike PBLs, BMLs mediated both NKC and ADCC, which were optimal at an E:T of 100:1. Similarly, in three experiments with a constant E:T of 100:1 and antiserum dilutions ranging from 1:10 to 1:1,000 (Fig. 5), the optimum ADCC was demonstrated with a final antiserum dilution of 1:10. Therefore, all subsequent experiments were performed with an E:T of 100:1 and a antiserum dilution of 1:10.

To determine whether BMLs were secreting antibodies or lymphokines during the incubation period and augmenting NKC in vitro (by causing ADCC or lymphokine-stimulated cytotoxicity), sterile, cell-free supernatants from bacteria-leukocyte assay mixtures were added to 50 μl of bacteria and 50 μl of fresh BMLs. In three experiments, NKC was unchanged in the presence of these supernatants (Table 7). Therefore, NKC was not augmented by in vitro secretion of immunoglobulins or lymphokines from BMLs.

Whole, nonadherent, and nylon wool-purified BMLs were used as effector cells at an E:T of 100:1 in paired experiments (Table 8). All three cell populations mediated NKC and ADCC. Therefore, removal of phagocytic cells by classic techniques did not decrease cytotoxicity. To determine whether the bactericidal activity was a result of intracellular or extracellular killing, 2 mg of phenylbutazone per ml was added to the assay, using whole and nylon wool-purified BMLs (Table 8). NKC and ADCC was significantly decreased by phenylbutazone ($P < 0.05$ in all cases) with both cell populations. However, NKC and ADCC were demonstrated even in the presence of 2 mg of phenylbutazone per ml. Thus BMLs appeared to mediate bactericidal activities via both intracellular and extracellular mechanisms.

TABLE 3. Characterization of lymphocyte effector cells

Expt	ADCC[a] of:				
	L[b]	ER[c]	NER[d]	Fc^{+}[e]	Fc^{-}[f]
1	30	0	43	ND	ND
2	47	ND	ND	45	0
3	45	0	33	41	0

[a] All experiments were performed with effector cells at an E:T of 100:1 in a 60-min bactericidal assay with a final antiserum dilution of 1:10. ND, Not performed.
[b] Overnight nonadherent lymphocytes were used as effector cells.
[c] E rosette-positive cells (T cells) were used as effector cells; >80% of lymphocytes were E-rosetting cells (ER).
[d] Non-E rosette cells were used as effector cells; <20% of lymphocytes were non-E-rosetting cells (NER).
[e] Fc receptor (EA rosette)-positive cells were used as effector cells; <20% were Fc^{+}
[f] Fc receptor-negative cells were used as effector cells; >80% of cells were Fc^{-}

DISCUSSION

Local immunity is critical to resistance in shigellosis. The actual mechanism of immunity remains unclear. Shigellosis is characterized by a dramatic inflammatory response (11, 45), and numerous fecal leukocytes are present in stools (9, 15, 37). Such a pronounced cellular response could lead one to speculate an important role for local cell-mediated immunity.

Lowell et al. (28) demonstrated ADCC against avirulent *S. flexneri* by human PBLs and immune serum. In contrast to the present study, these authors demonstrated ADCC by granulocytes as well as monocytes. Perhaps the differences are due to the higher E:T's used by Lowell and his coworkers (28). Experiments in our laboratory demonstrated that peripheral blood lymphocytes and whole mononuclear cells mediated ADCC, but not NKC, against virulent *S. sonnei*. Macrophages and polymorphonuclear leukocytes

TABLE 4. Effect of α Leu-7 and complement on lymphocyte cytotoxicity

Effector cells	Expt	Anti-*Shigella* cytotoxicity ADCC	Anti-HSV-1 cytotoxicity	
			NKC[a]	ADCC
Lymphocytes[b]	1	27	21	21
	2	35	77	63
Lymphocytes plus α Leu-7[c]	1	70	9	10
	2	43	60	45
Lymphocytes plus C[d]	1	30	23	19
	2	30	73	59
Lymphocytes plus α Leu-7 and C[e]	1	77	6	11
	2	45	63	43

[a] Calculated NKC against HSV-1-infected cells.
[b] Untreated lymphocytes; >90% viability by trypan blue exclusion.
[c] Lymphocytes which had been incubated with 60 μg of α Leu-7 for 1 h at 4°C; >90% viability by trypan blue exclusion.
[d] Lymphocytes that had been incubated for 1 h at 37°C in medium containing a final complement dilution (C) of 1:6; >90% viability by trypan blue exclusion.
[e] Lymphocytes that had been incubated with 60 μg of α Leu-7 for 1 h at 4°C; after centrifugation, the antibody-coated cells were resuspended in medium containing a final complement dilution (C) of 1:6; 70 to 75% viability by trypan blue exclusion.

TABLE 5. Effect of phenylbutazone on leukocyte bactericidal activity

Target cell	Effector cell	Leukocyte bactericidal activity (CFU/ml ± SE)[a]	
		With phenylbutazone[b]	Without phenylbutazone
S. sonnei 53GI	None	110.0 ± 17.2	107.5 ± 15.6
	Lymphocytes	111.0 ± 18.2	103.0 ± 14.1
	Lymphocytes plus antiserum	68.0 ± 15.1	71.0 ± 17.2
	ADCC[c]	39.5 ± 3.5	37.0 ± 4.0
E. coli ATCC 25922	None	108.5 ± 3.5	105.5 ± 2.5
	Polymorphonuclear cells	106.0 ± 4.0	105 ± 5.6
	Polymorphonuclear cells plus serum[d]	100.5 ± 0.5	70.0 ± 3.0
	ABI[e]	6.5 ± 1.5	34.5 ± 0.5

[a] CFU ($\times 10^3$) per milliliter from a 60-min bactericidal assay with an E:T of 100:1 and a final antiserum dilution of 1:10.
[b] All components were suspended in medium containing 2 mg of phenylbutazone per ml.
[c] Calculated ADCC. Mean ± standard error of two experiments.
[d] Bacteria were preopsonized with normal human serum by incubation at 37°C for 60 min.
[e] Calculated antibacterial index; mean ± standard error of two experiments.

failed to mediate NKC or ADCC. Killing by phagocytosis was not seen, probably due to the low number of phagocytes present in the assay and the absence of complement (2, 27). In vitro ADCC was optimum in a 60-min bactericidal assay with a high concentration of antiserum (in the absence of complement) and an E:T of 100:1. NKC was not demonstrated against *Shigella* spp. when peripheral blood effector cells were used.

The specificity of the anti-*Shigella* serum was examined by studying the PBL ADCC against other strains of *S. sonnei* and strains of *S. flexneri*. Three additional virulent strains of *S. sonnei* demonstrated susceptibility to lysis by ADCC. Two strains of virulent *S. flexneri* that were tested were not susceptible to lysis. Similarly, all three avirulent strains of *S. sonnei* were resistant to ADCC. These results suggest that the antiserum is directed against the somatic antigen of group D *Shigella* spp. since avirulent *S. sonnei* lack this surface structure and other species of virulent shigellae possess different surface somatic antigens (11).

The mechanism of PBL ADCC against virulent *S. sonnei* was further studied. It was our intent to determine whether the killing was via an intracellular or extracellular mechanism and whether a metabolic burst was required. Phenylbutazone inhibits intracellular killing of microorganisms without interfering with the binding of leukocytes and bacteria (26, 27, 43). Lymphocyte ADCC against *S. sonnei* was not affected by the presence of phenylbutazone, but killing of *E. coli* by polymorphonuclear leukocytes (in the presence of

TABLE 6. PBL antibody-dependent cellular cytotoxicity against *S. sonnei* 53GI

Source of effector cells	Mononuclear		Polymorphonuclear	
	n^b	ADCC[c] (mean ± SE)	n^b	ADCC[c] (mean ± SE)
Adults (>21 yr)	7	25.4 ± 3.5	5	12.2 ± 6.3
Children[d]	2	21.0 ± 7.1	ND	ND
Neonates[e]	5	53.8 ± 17.1	5	57.6 ± 14.8
CGD patient[f]	1	27.0	2	62.0 ± 20.2

[a] Cells were used as effector cells at an E:T of 100:1. ND, Experiments not performed.
[b] Number of experiments.
[c] Calculated ADCC, using immune serum directed against the homologous strain and an E:T of 100:1. $P < 0.05$ when the ADCC of adult and neonatal mononuclear and polymorphonuclear cells were compared, using a two-tailed, nonpaired t test.
[d] Leukocytes were collected from healthy children of ca. 1 year of age.
[e] Leukocytes were collected from cord blood after delivery.
[f] Leukocytes were collected from a patient with CGD.

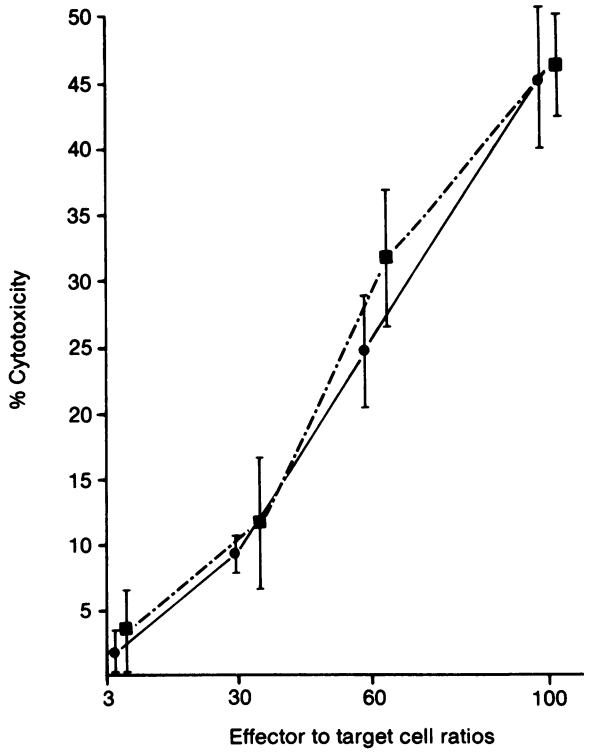

FIG. 4. Percentage of NKC (●) and ADCC (■) against *S. sonnei* with BMLs at four E:T's in a 60-min bactericidal assay, using a 1:10 dilution of normal rabbit serum (NKC) or immune rabbit serum (ADCC). The mean and standard error of three experiments are shown.

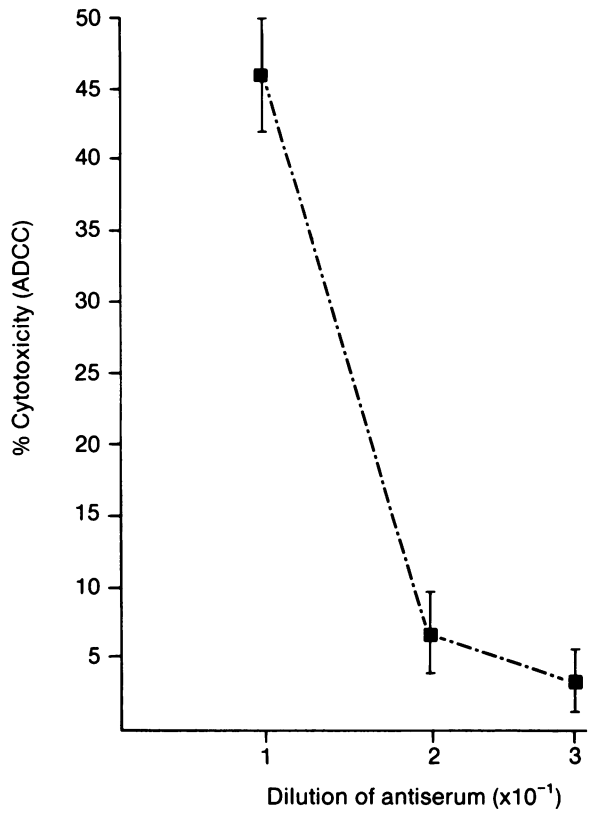

FIG. 5. ADCC against *S. sonnei* with BMLs at an E:T of 100:1 and dilutions of antiserum ranging from 1:10 to 1:1,000. The mean and standard error of three experiments are shown.

complement and normal human serum) in a standard phagocytosis assay was inhibited. These results further indicate that the bactericidal activity against *Shigella* spp. was not a result of phagocytosis and provide evidence for the idea that the cytolysis of virulent *S. sonnei* occurred extracellularly. Further evidence is provided when the anti-*Shigella* cytotoxic capabilities of leukocytes from a patient with CGD were examined. Leukocytes from patients with CGD are unable to kill most intracellular organisms. However, the mononuclear cells of the CGD patient mediated ADCC to a level that was not different from that of normal adults. The polymorphonuclear leukocytes of the CGD patient also mediated anti-*Shigella* ADCC, in contrast to experiments which demonstrated that polymorphonuclear cells from normal adults failed to mediate anti-*Shigella* ADCC. An additional conclusion which can be drawn from these data is that anti-*Shigella* ADCC, like classic antiviral ADCC, does not appear to require a metabolic burst since the killing defect in leukocytes from CGD patients is a failure to activate the respiratory burst and to produce bactericidal oxygen metabolites.

Experiments were performed to determine whether NK-like peripheral blood lymphocytes were the effector cells of anti-*Shigella* ADCC. The experiments demonstrated that non-T-cell lymphocytes with Fc receptors were the effector cells. Depletion of HNK-1$^+$ lymphocytes by complement-dependent cytotoxicity failed to inhibit anti-*Shigella* cytotoxicity. These results indicate that anti-*Shigella* ADCC is mediated by a NK-like lymphocyte other than a classic HNK-1$^+$ cell.

Cytotoxicity defects have previously been demonstrated in neonates and infants (1, 22). Therefore, we chose to examine the ability of leukocytes from neonates and infants to kill *Shigella* spp. There was no defect in neonatal or infant anti-*Shigella* ADCC mediated by mononuclear cells. In addition, polymorphonuclear leukocytes from neonates were able to mediate ADCC, similar to ADCC mediated by leukocytes from a CGD patient. Perhaps a compensatory immune mechanism exists in these populations where other immune deficiencies have been described.

Recently investigators have demonstrated both NKC and ADCC against enteric pathogens by using murine effector cells (34, 44). Gut-associated effector cells and secretory IgA were mediators of both NKC and ADCC against a *Shigella* sp.-*E. coli* hybrid (44). These results correlate with the present study in which colostral leukocytes, which are gut-derived cells, mediated both NKC and ADCC. Breast milk cells arise from progenitor cells in the Peyer patches of the small intestine and differ from those of the peripheral blood (9, 46). Nonadherent peripheral blood mononuclear cells consist of more than 90% lymphocytes, whereas nonadherent breast milk cells consist of a high ratio of macrophages. Only 5% of the total BML population are lymphocytes, in contrast to approximately half of the total PBL count. Despite the high percentage of phagocytic cells, colostral leukocytes are not efficient mediators of phagocytosis (17, 36). Previous studies from our laboratories have demonstrated that colostral leukocytes are poor mediators of NKC and ADCC against eucaryotic target cells (20, 21).

Since shigellosis is a localized enteric infection, gut-associated immunity would be expected to play a greater role than do systemic humoral immune factors. In the present study, we found that the anti-*Shigella* cytotoxicity of breat milk cells differs from that of PBLs. Peripheral blood lymphocytes were able to mediate ADCC, but not NKC, against virulent *S. sonnei*. BMLs were able to mediate both NKC and ADCC against virulent *S. sonnei* in an in vitro cytotoxicity assay. This difference is not unexpected since BMLs arise from progenitor cells in the gut. Gut-derived cells have been demonstrated as effective mediators of antibacterial NKC (34, 44). When phagocytes were removed by adherence to plastic or nylon wool, BMLs still mediated NKC and ADCC. The addition of phenylbutazone (an agent which inhibits intracellular bactericidal mechanisms) decreased, but did not ablate, the NKC and ADCC of breast milk cells.

In conclusion, peripheral blood lymphocytes anti-*Shigella* ADCC was mediated by a subpopulation of NK cells or other lymphocytes, which lack the HNK-1 surface antigen. These effector cells kill extracellularly in the absence of oxidative metabolism. Recognition may rely on carbohydrate moieties on bacterial cells, such as somatic antigens or other cell surface structures. BMLs were able to destroy virulent *S. sonnei* in the presence and absence of antibody via both intracellular and extracellular mechanisms. It is

TABLE 7. Augmentation of BML NKC against *S. sonnei* 53GI by in vitro secretion of immunoglobulins

Assay mixture	NKC[a]
BML + 53GI[b]	42.0 ± 6.5
BML + 53GI + BML − SUP[c]	44.3 ± 4.8

[a] Calculated NKC ± standard error of three experiments.
[b] BML plus normal rabbit serum was added to 10^5 CFU of *S. sonnei* per ml.
[c] BML plus normal rabbit serum was added to 10^5 CFU of *S. sonnei* per ml; after 60 min at 37°C, the suspension was filter sterilized and added to fresh BML-normal rabbit serum plus 10^5 CFU of *S. sonnei* per ml. Sup, Sterile, cell-free supernatants.

TABLE 8. BML cytotoxicity against *S. sonnei* 53GI

Effector cells	n[a]	BML cytotoxicity (± SE)			
		Without phenylbutazone		With phenylbutazone[b]	
		NKC	ADCC	NKC	ADCC
Whole[c]	8	39.4 ± 9.8	45.2 ± 10.4	27.0 ± 5.9	24.7 ± 8.2
Nylon wool[d]	3	31.7 ± 3.6	34.0 ± 2.6	20.7 ± 1.0	23.7 ± 2.4
Nonadherent[e]	5	45.6 ± 10.2	47.8 ± 10.2	ND[f]	ND[f]

[a] Number of experiments.
[b] Assay was performed in the presence of 2 mg of phenylbutazone per ml.
[c] Unseparated BMLs were used at an E:T of 100:1.
[d] BMLs which passed through double nylon wool columns were used at an E:T of 100:1.
[e] BMLs which did not adhere to plastic petri dishes were used at an E:T of 100:1.
[f] Experiments not performed due to an inadequate number of cells.

possible that these cells function in the gut of infants to protect them against shigellosis. These antibacterial mechanisms against *Shigella* spp. may be an important defense against infection in humans. The gut has an abundance of lymphocytes which have been shown to be capable of mediating cytotoxicity (3, 46). In addition, immunoglobulins and immunoglobulin-secreting cells are abundant, whereas complement is absent (40). Secretory IgA, the most abundant class of immunoglobulins found in the gut, is capable of mediating antibacterial ADCC (29, 44). Therefore, fecal and colostral antibodies and cells may play a role in in vivo NKC and ADCC.

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