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Helminthotoxic Responses of Intestinal Eosinophils to *Trichinella spiralis* Newborn Larvae

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Because the gastrointestinal lamina propria is the first line of defense against invasion with *Trichinella spiralis* newborn larvae, we investigated the helminthotoxic characteristics of isolated lamina propria eosinophils. Eosinophils were isolated from the intestinal lamina propria of rats and purified to nearly 90% purity by a combination of velocity sedimentation through Percoll and unit gravity sedimentation through a continuous gradient of bovine serum albumin. Isolated eosinophils were of high viability and responded to surface receptor stimulation. Freshly isolated intestinal eosinophils lacked cytotoxic capacity when incubated with newborn larvae in the presence of specific antiserum. Peritoneal eosinophils from the same rats exhibited 100% helminthotoxicity after 24 h. Cytotoxicity could be stimulated in the intestinal eosinophils by the addition of recombinant murine interleukin-5.

The intestinal epithelium is usually considered the first line of defense against invasive organisms in the intestinal tract. In the case of infection with *Trichinella spiralis*, this is clearly true for the infective larvae (27). *T. spiralis* newborn larvae (NBL), however, are born within the epithelial layer (8) and gain access to the host via the lamina propria vasculature (24, 25). The NBL leave the intestinal tract and migrate throughout the host, finally lodging in striated muscle, where they result in the pathology associated with trichinosis (11). It is at the point where the NBL are leaving the epithelial sheet and traversing the lamina propria to reach the local vasculature that cell-mediated immune responses could have profound helminthotoxic effects. In vitro data obtained by examining cells of the myeloid lineage, including eosinophils, from other sites (1, 10, 12) suggest that considerable NBL killing could be occurring at this site. Circulating eosinophils and peritoneal eosinophils (PEO) have been demonstrated to possess helminthotoxic and cytotoxic activity to a variety of parasites, cells, and tissue targets (5, 9, 22), predominantly by antibody-dependent cell-mediated cytotoxicity (ADCC). A variety of toxic eosinophil products, such as eosinophil major basic protein, eosinophil cationic protein, and eosinophil peroxidase, contribute to these cytotoxic effects (9, 26). Until now the contribution of intestinal eosinophils to these ADCC reactions has been impossible to ascertain, since cell preparations enriched for eosinophils have not been available for examination. The presence in the intestinal eosinophils of similar mediators, however, suggests that these cells could act in a similar manner and contribute significantly to local cell-mediated immune responses to *T. spiralis* NBL. Indeed, although many cells of the myeloid lineage will damage NBL, eosinophils are particularly effective at killing NBL less than 2 h old (7). We describe here the isolation and purification of intestinal eosinophils and the assessment of their toxic activity against *T. spiralis* NBL.

MATERIALS AND METHODS

Rats. Male 180- to 200-g Sprague-Dawley rats purchased from Charles River Canada Inc. were given food and water ad libitum.

Peritoneal cell isolation and enrichment. Peritoneal cells were obtained as previously described (14). Briefly, euthanized rats were injected with 15 ml of ice-cold *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered Tyrodes solution (HT; pH 7.4, 300 mosmol/kg) supplemented with 0.1% bovine serum albumin (BSA; Sigma), directly into the peritoneal cavity. After 2 min of gentle massage, the peritoneum was exposed and the lavage was collected with polyethylene pipettes and placed in cooled (4°C) tubes. Cells were washed twice with HT, counted, and assessed for viability by Trypan blue (0.1%; GIBCO) exclusion.

PEO were obtained by layering not more than 50×10^6 lavage cells in 10 ml of RPMI 1640 (GIBCO; in all cases supplemented with 10% fetal bovine serum, 10 mM HEPES, and antibiotics) on a discontinuous density gradient of Percoll (Pharmacia). Gradients were formed in clear polystyrene 50-ml tubes and consisted of a bottom layer of 10 ml of 65% Percoll (in RPMI) and an upper layer of 20 ml of 55% Percoll (in RPMI). The tubes were spun at $650 \times g$ for 45 min (4°C).

The cells from the 55%-65% Percoll interface were resuspended gently in RPMI and depleted of adherent cells by incubation in 25-cm^2 tissue culture flasks for 1 h at 37°C. Nonadherent cells, which contained purified eosinophils, were removed by gentle washing of the plates with warm RPMI. All functional studies were performed immediately after cell purification.

Cytocentrifuge (Shandon) smears were stained with May-Grunwald and Giemsa stains (BDH) to assess the composition of the preparations. Cell composition is given as a percentage of viable cells based on the morphology and staining characteristics of eosinophils and other cells in the preparations.

Intestinal cell isolation and purification. The isolation procedure for suspensions of lamina propria eosinophils (LPEO) from intestinal mucosa was modified from techniques described previously (4, 14). Briefly, rats were killed by cervical dislocation, and the entire small intestine was rapidly isolated and flushed with ice-cold phosphate-buffered saline (PBS; pH 7.4, 300 mosmol/kg). After the Peyer's patches and mesentery were removed, the intestines were

cut into small pieces in cold (4°C) HEPES (10 mM)-buffered Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution (GIBCO; pH 7.3). The pieces were incubated (37°C with stirring) in 10^{-4} M EDTA (disodium salt; Sigma) in Hanks balanced salt solution for three 10-min periods, separated by washes in warm Hanks balanced salt solution.

After the EDTA incubations, the tissue was incubated for 1 h in 25 U of collagenase (GIBCO) per ml in Hanks balanced salt solution supplemented with 20% fetal bovine serum. Cells obtained from this tissue digestion were filtered through sterile gauze and pelleted at 200 × *g* for 8 min at room temperature. Cells were resuspended in RPMI and cleared of coarse debris by rapid passage through a nylon wool column (300 mg of washed nylon wool in a 10-ml syringe barrel) at room temperature. The resulting cell suspension was centrifuged at 200 × *g* (10 min) and resuspended gently in RPMI (for further purification). The cell viability at this stage was determined as described above.

Enrichment for LPEO was achieved by mixing cells in 5 ml of RPMI with 5 ml of 100% Percoll (osmotically and pH balanced) to a final concentration of 50% Percoll (pH 7.4) and spinning at 400 × *g* (20 min, 10°C). The cell pellet was resuspended in 5 ml of RPMI, layered onto a 5-ml cushion of 68% Percoll (in RPMI), and spun at 400 × *g* (20 min, 10°C). The cells that did not enter the 68% Percoll cushion were washed and resuspended either in HT (for functional studies) or in PBS supplemented with 0.2% BSA (for further purification).

Further enrichment of LPEO was achieved based on cell size by using a unit gravity velocity sedimentation apparatus (STA-PUT; John's Scientific Co. Ltd.). Briefly, the Percoll-enriched cells, suspended in 15 ml 0.2% BSA (in PBS), were bottom loaded into the STA-PUT chamber, and a continuous BSA gradient (from 0.35 to 2%) was built from below. The cells were allowed to settle for 3 h at unit gravity (4°C). The separated cells were drained in 20 aliquots of 25 ml each in polystyrene centrifuge tubes. Each aliquot was spun for 5 min at 200 × *g* (4°C). Pellets were resuspended in cool (10 to 15°C) HT and counted. The viability and composition of the cell preparation were assessed as described above. All functional studies were performed immediately after separation.

Opsonization of zymosan. One milliliter of zymosan (Sigma; 4 mg/ml) in PBS was mixed with 1 ml of normal rat serum. The zymosan-serum mixture was incubated at 37°C for 1 h. Zymosan was separated from the serum by centrifugation (16,000 × *g*, 1 min, 20°C), washed twice in sterile PBS, and then resuspended to 2 mg/ml in PBS.

β-Glucuronidase release assay. Cells (2×10^5) were incubated in 500 μl of HT with or without a stimulus for 20 min at 37°C and then centrifuged (180 × *g*) for 10 min. The supernatants were separated from the pellets, and the pellets were resuspended to 500 μl with HT and then disrupted by freezing and thawing repeatedly in liquid N_2. Cell debris was removed from the lysed pellet by centrifugation, and aliquots (75 μl) were taken from both pellets and supernatants for the enzyme assay with 4-methylumbelliferyl-β-D-glucuronide (Sigma) as the substrate (3.78 mM). The substrate (75 μl) and 75 μl of the pellet or supernatant were incubated for 20 min at 37°C. The reaction was terminated with 1.5 ml of stopping buffer (0.1 M glycine, 32 mM NaOH [pH 10]). The calcium ionophore A23187 (Sigma) was used as a positive release control at a final concentration of 10^{-6} M.

Fluorimetric analysis was with a Perkin-Elmer (L5-3B) fluorimeter (excitation wavelength, 360 nm; emission wavelength, 450 nm). The percent release was calculated using the formula [supernatant/(supernatant + pellet)] × 100 (minus the spontaneous release).

TABLE 1. Purification of eosinophils from the rat peritoneal cavity by a combination of density centrifugation through Percoll and nonadherence to plastic

Cell prepn (*n*)	% Cell type (mean ± SD)			
	Eosinophils	Macrophages	Mast cells	Other cells
Peritoneal cells (4)	20.4 ± 2.7	53.3 ± 2.8	15.5 ± 4.6	10.6 ± 3.3
Percoll-enriched cells (7)	52.0 ± 16.0	39.0 ± 14.1	<1	7.6 ± 2.7
Nonadherent cells (6)	83.5 ± 5.5	14.8 ± 4.9	<1	1.4 ± 0.5

Parasites. The procedures for obtaining *T. spiralis* NBL have been described previously (13). Briefly, rats were killed 7 days after infection with 2,000 infective *T. spiralis* larvae. The intestines were removed, and nematodes were isolated by a modified Baermann technique. Adult worms were washed 10 times in sterile PBS and incubated in RPMI in 25-cm^2 tissue culture flasks overnight (37°C); then the flasks were emptied into 50-ml tubes, and the adult worms were allowed to settle. The NBL were harvested from supernatants by centrifugation (200 × *g*, 3 min).

Antibodies. Rats were given a primary infection with 5,000 *T. spiralis* infective larvae and after 12 days given a secondary infection with 2,000 infective larvae. Anti-*T. spiralis* infection serum was obtained by cardiac puncture 22 days after the secondary infection. Anti-*Nippostrongylus brasiliensis* infection serum was obtained from rats previously infected with 3,000 infective larvae (13). All references to serum volumes refer to neat (undiluted) serum.

Helminthotoxicity assay. NBL were washed 10 times in sterile RPMI, resuspended to 10^3 NBL per ml, and plated in 100-μl aliquots in 96-well flat-bottomed microtiter plates (Nunc). PEO and LPEO (10^6 cells per well) were incubated along with either anti-*N. brasiliensis* serum (50 μl), anti-*T. spiralis* serum (50 μl), or no serum with NBL. NBL were also incubated with anti-*T. spiralis* and anti-*N. brasiliensis* sera (50 μl) without cells. Cells were added in a volume of 100 μl of RPMI. The total volume per well was adjusted to 250 μl with RPMI when necessary.

Cell adherence was assessed by counting the percentage of larvae with more than 20 cells attached. Damage to worms was assessed according to the criteria of Mackenzie et al. (16) and consisted of microscopic examination of worm mobility and shape. NBL that were immobile and straightened were considered not viable.

Recombinant murine interleukin-5 (IL-5) was a generous gift of D. Kunimoto at the University of Alberta. Lyophylized IL-5 was reconstituted to 10,000 U/ml and used at concentrations from 500 to 2,500 U/ml. Recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF, Genzyme, Boston, Mass.) was obtained at a concentration of 5,000 U/ml and used at concentrations from 100 to 500 U/ml.

RESULTS

Purification of eosinophils from rat peritoneal cavity. Eosinophils constitute approximately 20% of peritoneal lavage cells in the rat (Table 1). Mast cells, macrophages, neutrophils, and lymphocytes constitute the other major cell types

TABLE 2. Purification of eosinophils from the rat lamina propria by a combination of density centrifugation through Percoll and sedimentation at unit gravity through BSA

Cell prepn (n = 4)	% Cell type (mean ± SD)			
	Eosinophils	Lymphocytes	Other cells	Mast cells
Lamina propria cells	20.3 ± 2.6	35.0 ± 4.2	46.8 ± 3.5	1.0 ± 1.3
After centrifugation through 68% Percoll	48.8 ± 10.9	18.4 ± 4.5	32.7 ± 8.3	0
STA-PUT purified	86.8 ± 4.7	3.9 ± 1.6	4.3 ± 2.9	0

in peritoneal lavage. Mast cells were the most dense and pelleted through 65% Percoll. In contrast, eosinophils did not penetrate the 65% Percoll cushion but did spin through 55% Percoll. Many macrophages, some neutrophils, and some lymphocytes did not enter 55% Percoll. The interface of the 55% and 65% Percoll gradient, therefore, was enriched for eosinophils (52% ± 16%, n = 7; Table 1). The principal contaminant of this interface population was the macrophage (39% ± 14.1%). Incubation of the interface population for the removal of plastic-adherent cells yielded a final purity of 83.5% ± 5.5% (n = 6) eosinophils.

LPEO isolation. Our initial experiments in isolating cells from the rat lamina propria indicated that, after the epithelial cells were removed with EDTA, collagenase digestion of the tissue resulted in the liberation of most of the LPEO earlier than the release of most of the mast cells. We took advantage of this fact and incubated the intestinal tissue for 1 h in bacterial collagenase. This regularly (n = 15) yielded approximately 40×10^6 cells per rat intestine. The viability of cells at this stage was always greater than 70% as determined by Trypan blue exclusion. Eosinophils represented 20.3% ± 2.6% of the total cells (Table 2).

After initial isolation, centrifugation through Percoll was performed to enrich for eosinophils on the basis of cell density. Numerous discontinuous gradients were tried, but eosinophil recoveries and enrichments were low. It was clear, however, that LPEO were more heterogeneous in density than PEO, because some LPEO would not pass through 55% Percoll but others passed through 65% Percoll. The maximum recovery of cells and enrichment for eosinophils were obtained by using a two-step procedure. First, the lamina propria population was mixed with Percoll to a concentration of 50%. The pellet from this centrifugation had enhanced viability (always >95%) and was depleted mainly of dead and dying epithelial cells. Recovery of total viable cells was routinely >80%. After this step the cell preparation was layered on a cushion of 68% Percoll. Centrifugation depleted the cell preparation completely of mast cells and erythrocytes and many lymphocytes. Eosinophils composed 48.8% ± 10.9% (n = 4) of the cells excluded from the 68% Percoll (Table 2). Recoveries were $5.3 \times 10^6 \pm 1.9 \times 10^6$ LPEO per rat intestine.

After enrichment on the basis of cell density, LPEO were further purified on the basis of cell size by utilizing unit gravity velocity sedimentation. No less than 10^7 Percoll-enriched cells were loaded on the STA-PUT apparatus. After 3 h, the STA-PUT was drained, and each fraction was assessed (by examination of stained cytocentrifuge smears) for eosinophils concentration. The maximum eosinophil concentrations occurred in fractions 12 through 15; the fraction with the highest recovery and percentage of eosinophils varied slightly from experiment to experiment. This procedure resulted in an eosinophil enrichment to 86.8% ± 4.7% (n = 4). The highest enrichment achieved in any particular experiment was 91%.

At this stage the viability determined by trypan blue exclusion was always greater than 90%. In addition, as reported below, the spontaneous release of the intracellular enzyme β-glucuronidase was always lower than 5%, substantiating the trypan blue exclusion estimates of viability.

β-Glucuronidase release assay. To assay the functional capabilities of the purified eosinophils, the release of the granule-associated enzyme β-glucuronidase in response to a stimulus was assessed. Both the calcium ionophore A23187 and opsonized zymosan, which mediates receptor-mediated exocytosis, were utilized. In both enriched PEO and LPEO populations, the spontaneous release of β-glucuronidase was less than 5%. There was no significant difference between LPEO and PEO in the total amounts of β-glucuronidase activity per cell as assessed by this assay. Both groups of cells were responsive to noncytotoxic levels of A23187 (10^{-6} M), although the LPEO were substantially less responsive than the PEO to this nonspecific stimulus. The β-glucuronidase release from LPEO was approximately 40% of that from PEO. The release initiated with opsonized zymosan as a biological stimulus showed a pattern similar to that of the nonbiological stimulus; the release of β-glucuronidase by the LPEO was substantial but still lower than the release by the PEO (Fig. 1). When receptor-mediated release induced by opsonized zymosan is expressed as a percentage of the release induced by the nonspecific stimulus (A23187), however, the release is similar in both LPEO and PEO (approximately 25%).

To control for the effects of the isolation procedure on intestinal eosinophil responsiveness, PEO were subjected to identical incubation with EDTA and collagenase and the STA-PUT purification procedure. In addition, peritoneal cells were incubated with cell-free digest material harvested from EDTA and collagenase digestion of the intestinal fragments (to control for possible deleterious effects of enzymes released from the digesting intestinal fragments). None of these methodologies reduced PEO responses to A23187 or opsonized zymosan by more than 15% (Fig. 1), indicating that the isolation and purification procedure is unlikely to be responsible for the reduced responsiveness in the LPEO.

Cell adherence and cytotoxicity to NBL in vitro. Killing of NBL by a mixed population of peritoneal lavage cells was utilized as a known positive control in our hands. PEO and LPEO were tested in a similar manner and assessed for adherence and killing after 18 and 24 h. In the case of LPEO, fractions 12 through 15 were tested in all cases to assure that selection of a specific subpopulation of eosinophils did not occur. We and others (13, 16) have previously shown that both adherence and killing of NBL by peritoneal cells are antibody dependent, so both eosinophil types were tested for killing in the presence of immune serum, normal rat serum, and antiserum raised to the nonrelated nematode *N. brasiliensis*. Adherence (and killing) occurred only in the presence of anti-*T. spiralis* antiserum and cells (Table 3). The immune serum itself was not cytotoxic (data not shown), and no cell adherence or killing was seen when normal rat serum or anti-*N. brasiliensis* serum was used. Complement was not required; heat inactivation of the immune serum (56°C, 30 min) made no difference to the adherence or killing.

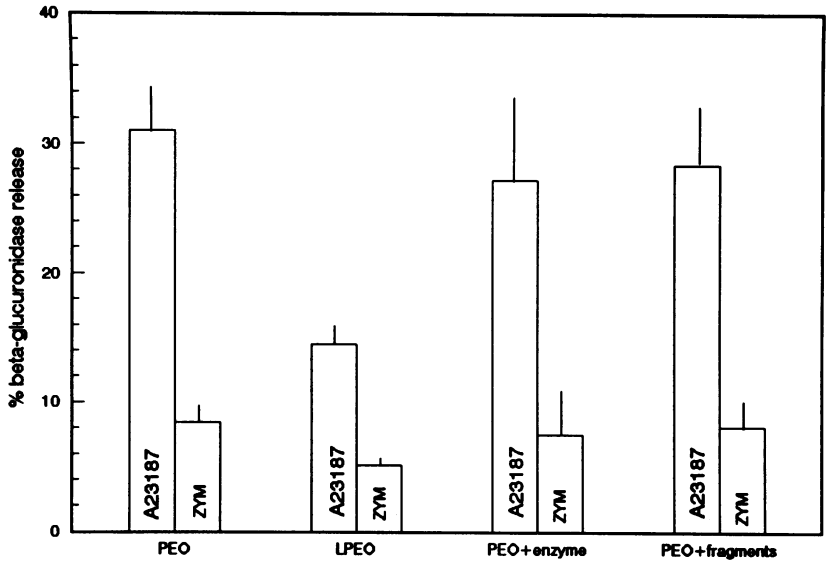

FIG. 1. Release of β-glucuronidase from PEO and LPEO stimulated by the calcium ionophore A23187 or opsonized zymosan (ZYM). PEO+enzyme and PEO+fragments represent release of β-glucuronidase from PEO that have been incubated with EDTA plus collagenase and with supernatants of digesting intestinal fragments, respectively, to control for the techniques used in the preparation of the LPEO.

LPEO, although able to adhere to NBL, lacked helminthotoxic activity. In contrast, substantial NBL killing by PEO was seen by 18 h, and all NBL were killed after incubation with PEO for 24 h (Table 3). The killing activity of PEO was reduced only slightly by identical EDTA and collagenase treatment or by incubation with cell-free digest material (Table 3), again indicating that the isolation procedure did not appear to be responsible for the lack of helminthotoxicity of the LPEO.

Since the cytotoxicity assay is dependent on cell viability over a longer period of time than is the β-glucuronidase release assay, control experiments were performed (n = 4) to assess the viability of LPEO and PEO over the period of the experimentation. After 24 h in culture, the PEO were 89% ± 4% viable and the LPEO were 82% ± 6% viable by trypan blue exclusion. Spontaneous release of β-glucuronidase was slightly higher (14% ± 5%) in the LPEO than in the PEO (11% ± 4%).

The addition of the stimulatory cytokine IL-5 increased the killing activity of the LPEO markedly. The results of a representative experiment (n = 5 for these experiments; each data point represents a mean of triplicates) are shown in Fig. 2. LPEO were capable of killing 22% ± 2% or 29% ± 6% of the NBL by 24 or 48 h, respectively, in the presence of 2,500 U of IL-5 per ml. Lower levels of IL-5 (500 and 1,000 U/ml) had limited (<10%) stimulatory activity (data not shown). Recombinant murine GM-CSF did not have a significant stimulatory effect (Fig. 2) when used at concentrations up to 500 U/ml and did not significantly enhance the stimulation resulting from IL-5 alone.

In all cases, cells attached to NBL were checked by differential interference contrast microscopy to confirm eosinophilic morphology.

DISCUSSION

In this study, we address the question of whether local intestinal cells of the myeloid lineage, in this case eosinophils, have profound anti-parasite activities against the NBL of *T. spiralis*. Such cells could theoretically function to block NBL access to the vasculature and result in their death in the lamina propria. Evidence for such intestinal killing of migrating larvae has been found in studies of pigs with naturally developed immunity to the nematode *Ascaris lumbricoides*

TABLE 3. Assessment of adherence to and killing of NBL by PEO and LPEO

Serum[a]	Time (h)	Mean (range) % adherence to NBL[b]			Mean (range) % killing of NBL[c]			
		Peritoneal cells	PEO	LPEO	Peritoneal cells	PEO	LPEO	Enzyme-treated PEO
IS	18	100 (0)	94 (2)	72 (8)	64 (12)	51 (17)	0	46 (12)
IS	24	100 (0)	100 (0)	100 (0)	100 (2)	100 (0)	2 (1)	98 (4)
NS/NRS	18	0	0	0	0	0	0	0
NS/NRS	24	0	0	0	0	0	0	0
HI-IS	18	100 (0)	92 (3)	78 (7)	71 (14)	48 (9)	0	37 (11)
HI-IS	24	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	1 (4)	97 (5)

[a] IS, immune anti-*T. spiralis* antiserum; NS/NRS, immune anti-*N. brasiliensis* antiserum or normal rat serum; HI-IS, heat-inactivated immune anti-*T. spiralis* antiserum.

[b] Percentage of larvae with >20 cells attached (n = 4).

[c] Percentage of larvae killed (n = 4 for all but enzyme-treated PEO [n = 2]).

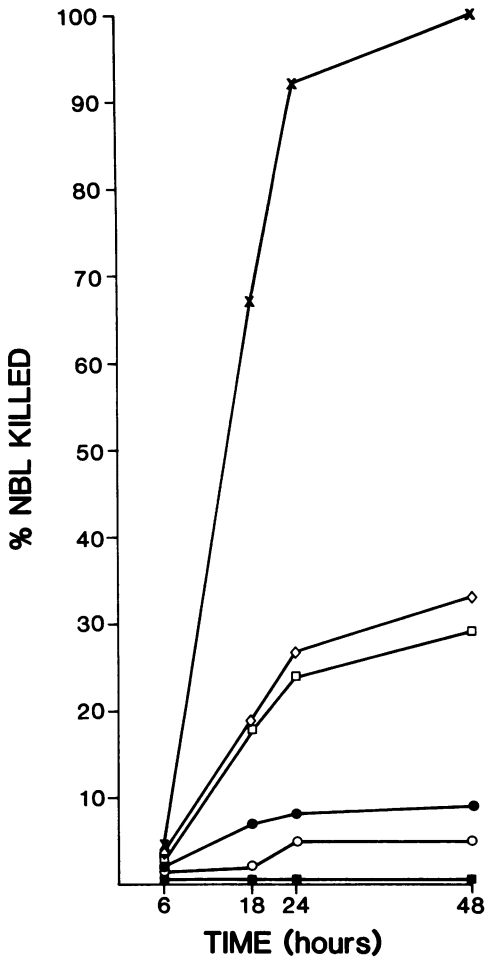

FIG. 2. Effect of recombinant murine IL-5 and GM-CSF on helminthotoxicity of LPEO. ×, PEO; ◇, LPEO plus 2,500 U of IL-5 and 500 U of GM-CSF per ml; □, LPEO plus 2,500 U of IL-5 per ml; ●, LPEO plus 500 U of GM-CSF per ml; ○, LPEO without cytokines; ■, no cells (NBL alone).

(21). What has been lacking is direct evidence of in vitro killing by intestinally derived granulocytes. This report describes experiments in which enriched populations of intestinal eosinophils were obtained and tested for helminthotoxic activity against *T. spiralis* NBL.

We isolated eosinophils from the lamina propria of normal rats by collagenase digestion of intestinal tissue. Enrichment of the isolated lamina propria population for eosinophils entailed, first, centrifugation over a discontinuous density gradient of Percoll. After enrichment by cell density, the eosinophils were further enriched by separation on the basis of cell size by utilizing a STA-PUT apparatus at unit gravity. These procedures are modifications of procedures we have published previously for the purification of intestinal mast cells (14).

LPEO and PEO were compared as to their ability to release β-glucuronidase via surface receptor-mediated stimulation (with opsonized zymosan). Although, in general, the LPEO are less responsive to stimulation by A23187, at noncytotoxic levels, they show the same percent release (in comparison to A23187) to opsonized zymosan as the PEO, indicating that surface receptor-mediated release of β-glucuronidase in LPEO and PEO is comparable. β-Glucuronidase release was chosen as a measure of activation since this enzyme is an eosinophil granule component that has been confirmed, immunocytochemically, to be present in rat intestinal eosinophils (28) and is released under noncytotoxic conditions. The similarity in surface receptor-mediated release supports earlier suggestions that intestinal eosinophils possess surface receptors for antibody and complement (2). Surface receptors for antibody on LPEO are also confirmed by their antibody-dependent adherence to *T. spiralis* NBL (see below).

Eosinophils from the intestines of normal rats differ, however, from eosinophils from the peritoneal cavities of the same rats with respect to ADCC against *T. spiralis* NBL. These differences cannot be accounted for by the preparation techniques, because eosinophils isolated from the peritoneal cavity and subjected to methods of preparation that were as close as possible to those used for LPEO were not significantly effected in their cytotoxic potential. Previous experiments (13) with less pure populations of LPEO demonstrated that mixing the LPEO preparation with PEO had no effect on PEO function in the NBL cytotoxic assay, indicating that suppressive activity in the lamina propria preparation was not responsible for the lack of cytotoxicity. These earlier experiments also indicate that the enrichment procedure does not select for a subpopulation of eosinophils that are noncytotoxic, since even at high effector/target ratios the unenriched LPEO were not capable of killing. The differences in levels of killing in LPEO and PEO are also not due to the greater percentage of macrophages in the PEO population. Our reasons for concluding this are threefold: first, occasionally the PEO population was as pure as 95 to 97% eosinophils, and no difference was seen between killing with these cells and that with less pure (85% eosinophil) preparations; second, PEO killing of *T. spiralis* has been documented by other authors (17); third, microscopic examination of the cells in the PEO preparation adhering to NBL revealed that they were almost exclusively eosinophils. In addition, our previous experiments (13) have demonstrated that the whole peritoneal cell population, before enrichment, could be titered down to a level at which no killing is evident. At this point, the actual number of macrophages is similar to the numbers present as contaminating cells in the PEO population. Mixing of these cells with LPEO does not confer the ability to kill. This suggests that peritoneal macrophages are not capable of helping LPEO to kill, although it does not rule out the possibility that the peritoneal macrophages aid in PEO killing, an area which is currently being explored in our laboratory.

The reasons for the lack of helminthotoxicity by freshly isolated LPEO are unknown. It is clear that LPEO adherence is not as extensive as PEO adherence. In the LPEO wells, all NBL had >20 cells attached by 24 h of incubation but <5% of the worms had >50 cells attached. Rarely were the worms completely covered with cells. In contrast, however, all NBL in the PEO wells were completely covered with cells after 24 h. Cell adherence alone cannot, however, explain the lack of killing of LPEO, since some NBL were completely covered with LPEO and were not killed.

The presence of toxic eosinophil molecules such as major basic protein, eosinophil cationic protein, and eosinophil peroxidase have been demonstrated in intestinal eosinophils (20). Indeed, worm death has been demonstrated to result from incubation with lysates of intestinal lamina propria cells (6). We have reported (13) that a small amount (<10%) of killing is seen after 48 h incubation with less pure popula-

tions of LPEO. Extended incubation (for 6 days) with these unenriched cell populations, to a point where loss of eosinophil viability occurs, results in the death of a significant number of larvae. One could therefore postulate that in the LPEO the activation of the killing mechanism is incomplete.

The primary signal for adherence is clearly antibody, as has been demonstrated in a number of other parasite systems (23), and is parasite specific (in that anti-*N. brasiliensis* serum does not induce adherence). Local lymphokines, or some aspect of the isolation procedure that we were unable to control for may, however, have rendered the LPEO unable to convert the Fc receptor signal into an effective ADCC response. The addition of recombinant murine IL-5, which is known to upregulate eosinophil activity (19), was able to partially but not completely overcome this block in ADCC activity. GM-CSF, although shown in other systems to increase cytotoxic activity of peripheral blood eosinophils (18), had little effect on LPEO cytotoxicity against NBL. There are at least two potential reasons why GM-CSF showed no activity in this system. First, it is possible that murine GM-CSF is not effective in this respect in the rat system. Second, although both native and recombinant GM-CSF are known to effect murine eosinopoesis (17), they have not been clearly demonstrated to have stimulatory effects on mature eosinophils that are similar to those of human GM-CSF.

T. spiralis NBL are biologically relevant targets for LPEO. Therefore, it was surprising to find that LPEO were ineffective at NBL killing and that stimulation with cytokines conferred only partial cytotoxic activity on these cells in comparison with the 100% cytotoxic activity of eosinophils from the peritoneal cavity of the same animals.

It is impossible to control completely for the effects of enzymatic digestion of tissue to obtain cells for in vitro experimentation, and the reduced responsiveness of the LPEO to stimulation with the calcium ionophore A23187 may represent some of those effects. The complete lack of ADCC activity in the LPEO, however, is unlikely to result entirely from these effects, especially since the PEO given identical enzymatic treatment or treated with cell-free digest material were only marginally reduced in their cytotoxic activity. It is possible that specific microenvironmental factors produced by other immunologically active cells modulate LPEO function in a lasting manner. Cytokines produced by lymphocytes, macrophages, and mast cells have been shown to influence eosinophil function in vitro (19) and may well contribute to the development of a local functional repertoire. This suggests that assumptions about eosinophil antiparasite functions in various tissues based on extrapolations of PEO function must be treated with caution. There is already clear evidence that mast cells with distinct properties exist at mucosal sites (3, 15). The NBL cytotoxicity data presented here may represent another example of the difficulties inherent in easy extrapolations regarding granulocyte function.

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