Recognition and Destruction of Bacillus Calmette-Guerin–infected Human Monocytes

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Summary

We have established a long-term culture system to study macrophages chronically infected with mycobacteria. Monocytes are infected with Bacillus Calmette-Guerin (BCG) and support exponential intracellular replication without profound perturbation of normal host cell function. We have used this system to investigate lymphokine-activated killer (LAK)-mediated cytolysis. We have found that interleukin 2 stimulation of peripheral blood lymphocytes generates a cytotoxic activity against human monocytes. A $CD56^-$ subpopulation of LAK cells specifically recognizes and lyses BCG-infected cells. Lysis of the host cell has no effect on parasite viability and results in the liberation of bacteria capable of infecting more cells.

The mechanisms of host resistance against pathogenic mycobacteria remain unclear after a century of investigation (1). Interest in this field has been stimulated by the increased worldwide incidence of these diseases and the emergence of multidrug-resistant organisms (2). Work from this laboratory has focused on *Mycobacterium leprae* and elucidated determinants that are associated with some of the immunodeficiencies expressed in lepromatous leprosy. These include the host's inability to generate a cell-mediated immune response to *M. leprae* antigens and the inability of cutaneous macrophages to kill this obligate intracellular parasite (3). Recovery from infection requires the host to destroy heavily parasitized, oxidatively incompetent macrophages and facilitate the bacterial uptake by freshly emigrated, bactericidal blood monocytes.

We now examine an in vitro model of mycobacterial infection in which we parasitize human blood monocytes with Bacillus Calmette-Guerin (BCG)[1] and quantitate the extent and rate of their intracellular replication. We report that an IL-2-induced LAK cell of a $CD56^-$ phenotype preferentially destroys infected monocytes.

Materials and Methods

BCG. The Pasteur strain 1011 of BCG (Trudeau Institute, Saranac Lake, NY) was grown for 7–8 d in spinning culture in endotoxin-free modified Proskauer-Beck medium and stored in liquid nitrogen at 10^9 CFU/ml. CFU were estimated by plating dilutions of sonicated bacterial suspension on Middlebrook and Cohn 7H9 agar plates that were incubated at 37°C for 2–3 wk before visual counting of colonies arising from single viable organisms. Cultures contained 30–40% bacteria capable of growing on solid medium. Bacteria were endotoxin free by the limulus amoebocyte lysate assay (Whittaker Bioproducts, Baltimore, MD).

Monocytes. PBMC from normal human tuberculin responders and nonresponders were isolated on Ficoll/Paque and depleted of T lymphocytes with neuraminidase- (Calbiochem-Behring Corp., La Jolla, CA) treated sheep erythrocytes (En) (Scott Laboratories, Friskeville, RI). The nonrosetted cells (En PBMC) were resuspended at a denstity of 6×10^6/ml in RPMI supplemented with 10% polled AB^+ human serum (Biocell, Carson, CA), of which 5 ml was plated on 100-mm tissue culture dishes (Corning Glass Works, Corning, NY) or 0.1 ml on Thermanox coverslips (Lux, Naperville, IL). After 1 h nonadherent cells were washed away and the enriched monocytes were cultured at 37°C in 5% CO_2 at a density of $\sim 2 \times 10^6$/ml in RPMI supplemented with 20% pooled AB^+ human serum (Biocell) and 100 μg/ml ampicillin (Sigma Chemical Co., St. Louis, MO). For infection some of the cultures were exposed to a single-cell suspension of BCG containing one to five viable bacteria per cell. After 6 d of culture, tissue culture plate monocytes for use as targets in cytotoxicity assays were gently scraped up into suspension with a rubber policeman. Approximately 50% of the cells originally plated were recovered after scraping and >90% of the labeled cells used as targets in cytotoxicity assays were viable by trypan blue exclusion.

Effector Populations. Effector populations were generated by coculturing En^+ PBMC for 5 d at 2.5×10^6/ml in RPMI supplemented with 10% pooled AB^+ human serum and 100 μg ampicillin/ml (unstimulated cells) and 1,000 U/ml rIL-2 (Cetus Corp., Emeryville, CA) (LAK cells). Antigen-driven cytotoxic cells were generated by coculture of PBMC with 10 μg/ml PPD (Statens Seruminstitut, Copenhagen, Denmark). For cell depletion 2 ×

[1] *Abbreviations used in this paper:* BCG, Bacillus Calmette-Guerin; En, neuraminidase-treated sheep erythrocyte.

10^7 LAK cells were incubated on ice with 10 μg Leu-19 (anti-CD56; Becton Dickinson & Co., Mountain View, CA) or TCR-δ1 (anti-δ chain of the TCR; a kind gift from Dr. M. Brenner [4]) for 1 h and washed three times. Cells bound by antibody were removed with two sequential rosetting steps with 6×10^7 Dynabeads M-450 (Dynal A. S., Oslo, Norway) coated with sheep anti-mouse IgG1 (Fc). Efficiency of depletion was assayed by FACS® analysis (Becton Dickinson & Co.).

Cytotoxicity Assay. 2–3 $\times$ 10^6 targets (monocytes or Daudi cells) were labeled in suspension with 200 μCi [^{51}Cr]sodium chromate in a volume of 200 μl for 1 h at 37°C, washed twice, and then 10^4 cells were incubated in triplicate with or without effector cells in U-bottomed 96-well tissue culture plates (Costar, Cambridge, MA). Some target cells were pulsed with 40 μg/ml PPD or with BCG during the assay as described. After 16 h at 37°C in 5% CO_2, supernatants were harvested (Skatron, Hunt Valley, MD) and ^{51}Cr release was counted. Percent specific ^{51}Cr release was calculated as: 100× [(cpm with effectors − cpm without effectors)/(cpm with 1% Triton − cpm without effectors)]. Results are expressed as the mean of triplicate wells ± SEM.

Cytotoxicity/CFU Assay. Monocytes (~2.5 $\times$ 10^5/coverslip) were labeled in situ on coverslips with 50 μCi [^{51}Cr]sodium chromate as above and washed by dipping in warm RPMI. Where relevant 2.5 $\times$ 10^6 effector cells (~10 cells/monocyte) were added in triplicate 1-ml wells. After a 16-h incubation culture medium was removed and monolayers were lysed in 1 ml 0.008% digitonin. 0.5-ml aliquots of culture medium and lysate were counted to estimate specific ^{51}Cr release. Percent ^{51}Cr release was calculated as: 100× [cpm in culture medium/(cpm in culture medium + cpm in lysate)], and from this percent specific ^{51}Cr release was calculated as: percent ^{51}Cr release with effector − percent ^{51}Cr release without effectors. In parallel, dilutions of the lysates and culture medium were plated on solid medium for CFU estimation as described above.

Apoptosis and Necrosis. ^{51}Cr-labeled monolayers of monocytes on coverslips were incubated with culture medium or culture medium containing 5 mM ATP or GTP (Boehringer Mannheim, Biochemicals, Indianpolis, IN) or 20 mM H_2O_2 (Fisher, Fair Lawn, NJ). At the times indicated specific ^{51}Cr release was calculated as above.

Acid-fast Staining. Monolayers of infected monocytes were fixed in formalin for 10 min, dried, and treated with phenolic 1.5% auramine/0.75% rhodamine for 10 min before extensive washing with acid alcohol followed by water. After drying and mounting cells were examined by phase contrast and acid-fast bacteria were visualized using a BG-12 exciter filter and an OG-1 barrier filter (Nikon Corporation, Tokyo, Japan).

FACS® Analysis. Cell populations were stained with mAbs (Becton Dickinson & Co.) directly conjugated with FITC or PE. After incubation on ice for 30 min, cells were washed three times, fixed with formalin, washed twice, and analyzed on a FACScan®.

Results

Monocytes in Culture Are Permissive for the Growth of BCG. When monocytes in culture were exposed to single-cell suspensions of BCG, the bacteria were efficiently internalized and could be visualized and enumerated by acid-fast staining. At the low multiplicity of infection (MOI) used in these experiments, between 40 and 70% of the cells were infected with single organisms. Less than 1% of infected cells contained more than two BCG per cell. BCG replicated intracellularly and growth was followed both by acid-fast staining and by colony counts on lysates of infected cells (Fig. 1). Extracellular growth and growth in medium without cells were negligible. After an initial delay in replication, the generation time for BCG within macrophages in this system was between 20 and 24 h, which is comparable to the generation time measured in optimal conditions of spinning culture. After 8 d of intracellular replication, when most infected cells contained ~100 bacteria, monocytes began to lyse. After 6 d of culture when cells were used as targets in cytotoxicity assays, 60–70% of the cells were infected and most infected cells contained 10–20 bacteria per cell (Fig. 2).

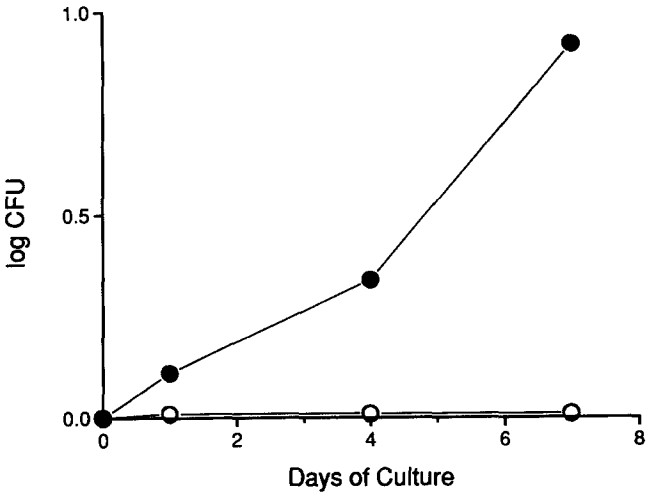

Figure 1. Monocytes in culture are permissive for the growth of BCG. Freshly isolated monocytes in monolayers were washed to remove nonadherent cells and exposed to about one BCG per cell in suspension. At the time points indicated the culture medium was removed from wells in octuplicate, the cells were lysed in 0.008% digitonin in water, and serial dilutions of both medium and lysate were plated out on solid 7H9 medium for CFU estimation (●) after 2–3 wk of incubation at 37°C. Wells without human cells were treated in parallel (○). In the presence of cells, <10% of the CFU are found in the medium at any time point, and values shown are the sum of medium and cell lysate. CFU in the medium were never >10% of the total. Results are expressed as log CFU, which is calculated as log CFU at the experimental time point minus log CFU immediately after infection. Data presented are from a representative assay performed >10 times.

We have compared a wide range of characteristics of infected and uninfected cells and the presence of multiplying intracellular BCG had few marked effects on any of the parameters of monocyte physiology studied, including gross morphology, surface antigen expression (CR3, CD14, CD16), profiles of phosphorylation and protein synthesis (in resting and IFN-γ-treated cells), and microbicidal activity. Although class II MHC expression was reduced on infected cells, class I expression on both populations was comparable. Both populations maintained similar viability in culture. By nuclear counts <30% of both populations were lost in 7 d. In addition, both populations exhibited similar resistance to lysis. If soluble mediators of necrosis (H_2O_2) or apoptosis (ATP^{4-}) were titrated into cultures, both uninfected and infected cells died over a similar dose range and with similar kinetics (Table 1). Necrotic and apoptic mechanisms of cell death were evalu-

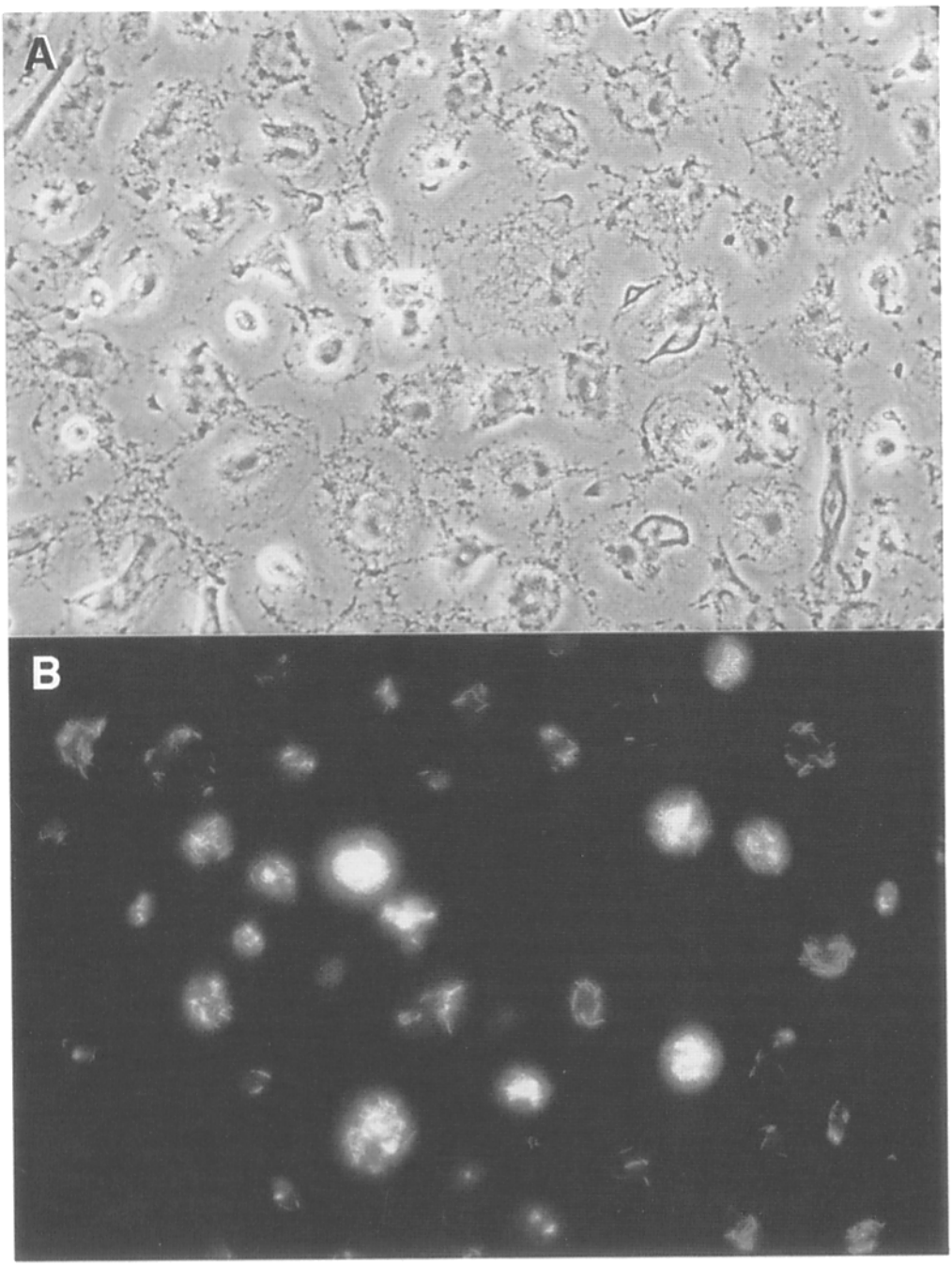

Figure 2. The morphology of infected monocytes. A monocyte monolayer on a coverslip was infected with about one BCG per cell and cultured for 7 d. The coverslip was removed, fixed in formalin for 10 min, and stained for acid-fast bacteria. Phase (*A*) and fluorescence (*B*) images of the same 40× field are shown. Infected cells appear healthy despite the presence of large numbers of intracellular bacilli.

ated by electron microscopy of the dying cells. Uptake and spontaneous release of sodium ^{51}[Cr]chromate by both populations were comparable; each incorporated 0.5–1.0 cpm per cell and spontaneously released 20–30% of incorporated radioactivity over 16 h. Most importantly, when pulsed with the relevant antigen both infected and uninfected cells were lysed to the same extent by antigen-educated CTL, suggesting that infected cells are not inherently more susceptible to cell-mediated cytolysis (Fig. 3).

LAK Cells Preferentially Recognize Infected Cells over Uninfected Cells. Coculture of PBMC with IL-2 resulted in the generation of LAK activity as detected by lysis of the NK cell–resistant Daudi cell line (Fig. 4 *A*). When a lytic activity was developed against the Daudi cell line a parallel lytic activity was developed against monocytes. Unstimulated effector cells had no activity but IL-2-activated effector cells killed monocytes in a dose-dependent fashion (Fig. 4 *B*). Although levels of lysis varied from assay to assay infected cells were consistently recognized and lysed more efficiently than uninfected cells. About 20% lysis of uninfected cells was seen at an E/T ratio of 25:1. A comparable level of lysis of infected cells was seen at a lower E/T ratio of ~6:1, therefore

Table 1. *Percent Specific ^{51}Cr Release in the Presence of Soluble Inducers of Apoptotic and Necrotic Cell Death*

Duration of treatment	Cells	Treatment			
		Medium	5 mM GTP	5 mM ATP	20 mM H_2O_2
h					
4	Uninfected	0	−2	14	26
	Infected	0	−5	13	22
20	Uninfected	0	1	40	56
	Infected	0	−1	34	52

infected cells were lysed about five times more efficiently than uninfected cells. Thus, within the IL-2-induced cytotoxic population there were two activities, on that lysed monocytes whether infected or not, and another that preferentially recognized infected cells.

LAK Killing of Infected Cells Fails to Reduce the Viability of Intracellular BCG. To probe the role of LAK-mediated cytotoxicity in controlling the growth of mycobacteria the effect of cytolysis on parasite viability was studied. Even when most (>90%) of the infected cells were killed no change in BCG viability was observed (Fig. 5). In this series of assays monolayers of adherent monocytes (as opposed to cells scraped up into suspension) were labeled and incubated with effectors. Under these conditions, where monocytes are more accessible than in microtiter wells, more efficient lysis is observed. The failure of LAK-mediated host cell lysis to affect BCG viability suggests that there is no microbicidal mechanism coupled to this host cell killing mechanism.

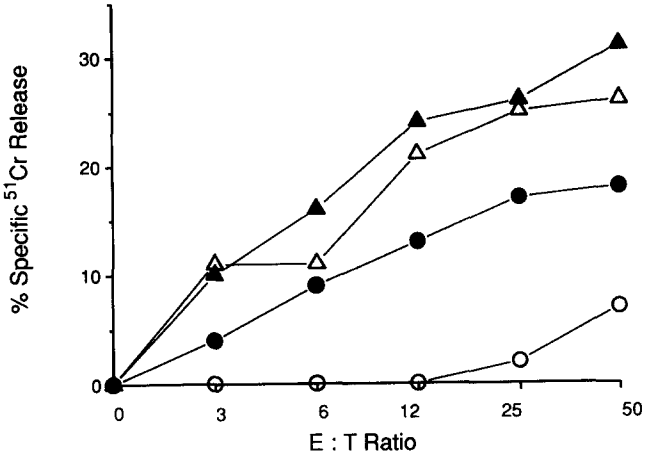

Figure 3. Antigen-dependent cell-mediated lysis of monocytes. 3–50 × 10^4 PBMC stimulated by culture in medium containing 10 μg/ml PPD were washed and incubated with 10^4 uninfected (*open symbols*) or infected (*filled symbols*) monocytes in the presence (▲ and △) or absence (● and ○) of 40 μg/ml PPD. PBMC not stimulated by culture with PPD did not lyse any target population (not shown). Infected cells were lysed by activated PBMC, whereas uninfected cells were not. Both populations were lysed by activated effectors with comparable efficiently in the presence of saturating antigen.

LAK Activity Is Manifest against Syngeneic and Allogeneic Targets. LAK cells lyse tumor cells by an MHC-independent mechanism. To begin to investigate the MHC restriction of the LAK activity against monocytes, the efficiency of lysis of syngeneic and allogeneic targets was compared. Target monocytes were lysed efficiently by both syngeneic and allogeneic LAK cells (Fig. 6), and infected cells were lysed more efficiently than uninfected cells regardless of the compatibility of the effector. In the data shown allogeneic targets were lysed more efficiently. However, in the parallel assay the targets from the same donor were lysed more efficiently by syngeneic

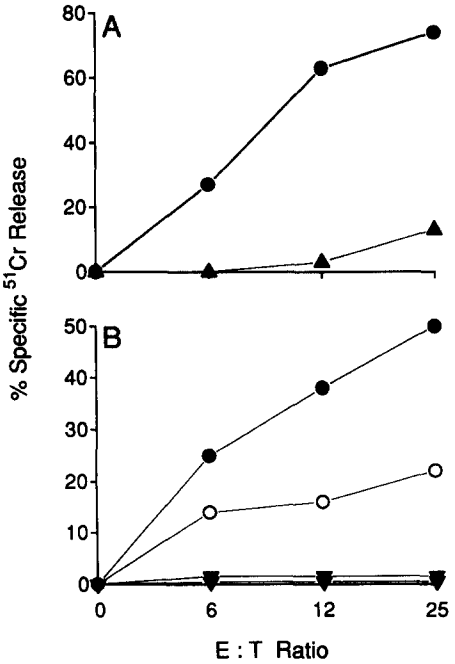

Figure 4. LAK cells lyse Daudi cells and monocytes and preferentially lyse infected monocytes. (*A*) 6–25 × 10^4 LAK cells (●) or unstimulated Er^+ PBMC (▲) were incubated with 10^4 Daudi cells. Only LAK cells induced significant ^{51}Cr release. (*B*) 6–25 × 10^4 LAK cells (● and ○) or unstimulated Er^+ PBMC (▼ and ▽) were incubated with infected (*filled symbols*) or uninfected (*open symbols*) monocytes. Infected monocytes were lysed by LAK cells more efficiently than uninfected cells. Data presented are from a representative assay performed >10 times.

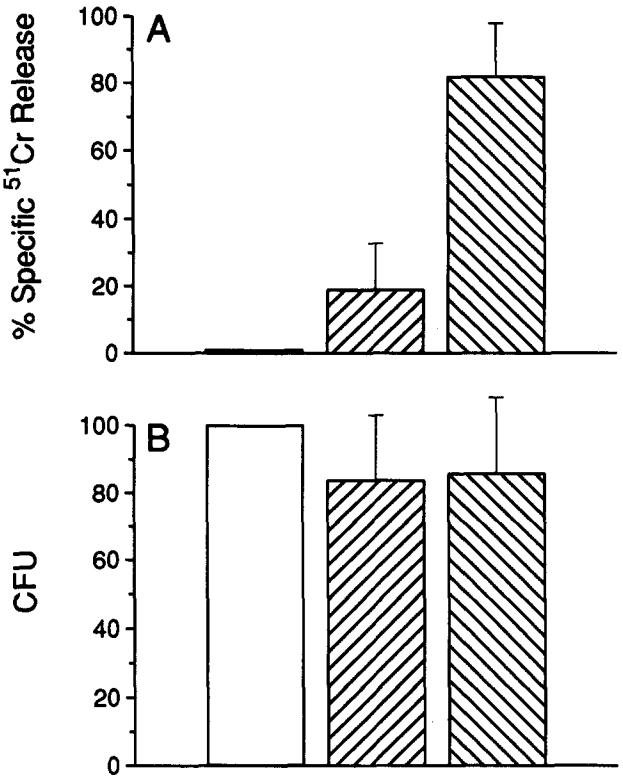

Figure 5. LAK killing of host cells fails to reduce the viability of intracellular BCG. About 2 × 10^5 monocytes were incubated on coverslips in culture medium alone (□), and with 2 × 10^6 Er^+ PBMC that had either been cultured for 5 d in culture medium alone (▨), or in culture medium supplemented with 1,000 U IL-2/ml (▩). After 16 h percent specific ^{51}Cr release (*A*) and CFU (*B*) were determined from parallel triplicate coverslips. Results are expressed as the ± SEM of six experiments.

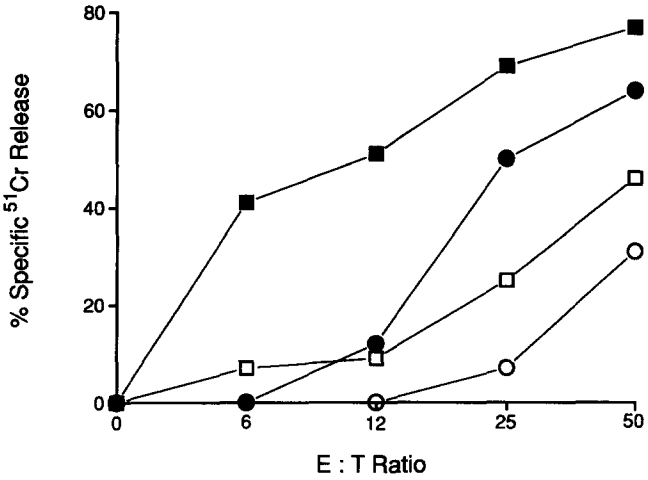

Figure 6. Efficient lysis is mediated by syngeneic and allogeneic LAK cells. 6–50 × 10^4 syngeneic (● and ○) or allogeneic (■ and □) LAK cells were incubated with 10^4 uninfected (*open symbols*) and infected (*filled symbols*) monocytes. Infected target cells were preferentially recognized by both syngeneic and allogeneic effectors. The results are of a representative experiment repeated three times with different donor pairs.

LAK cells, suggesting that in this assay this donor's monocytes were more susceptible to lysis. In general both the nonselective cytotoxic activity against monocytes and the selective recognition of infected monocytes were efficiently mediated by syngeneic and allogeneic LAK effector cells.

Selective Lysis Is Independent of Exogenous Mycobacterial Antigen. To investigate whether the preferential recognition of infected cells was due to the presentation of antigen produced endogenously by replicating BCG, uninfected target cells were pulsed with antigen during the assay. Exposure of uninfected cells to 40 μg/ml of PPD had no effect on LAK killing (Fig. 7). In a parallel assay this treatment with PPD sensitized monocytes for lysis by PPD-educated CTL (Fig. 3), suggesting that these monocytes were presenting antigen. Since there may be antigens produced by BCG not represented in PPD the effect of exogenously added BCG was also investigated. Target cells were pulsed during the assay (i.e., infected) with ~1 bacillus per cell or ~10 bacilli per cell. The lower dose represents the initial infecting dose and the higher dose reproduces the bacillary load in infected cells after 6 d of intracellular replication, i.e., at the time of assay. Both pulses are sufficient to sensitize monocytes for lysis by PPD-educated CTL. The BCG pulse had no effect on LAK activity against either target population and most notably failed to augment lysis of hitherto uninfected cells to the levels seen with chronically infected cells. We conclude that recognition of infected cells does not depend on presentation of mycobacterial antigen and is a property of long-term, but not transient, infection with viable bacilli. Incubation of target cells at 42°C for 60 min immediately before addition of effectors failed to affect the efficiency of lysis of either uninfected or infected monocytes (data not shown), suggesting

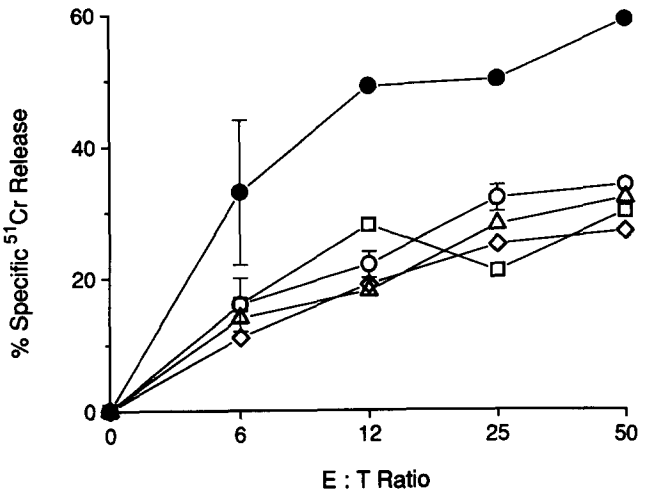

Figure 7. LAK activity is independent of exogenous mycobacterial antigen. 6–50 × 10^4 LAK cells derived from a PPD-responsive donor were incubated with 10^4 infected cells (●), or uninfected cells in the absence (○) or presence (△) of 40 μg PPD/ml or presence of ~10^4 BCG (◇) or ~10^5 BCG (□). Presentation of PPD or transient infection with BCG did not render monocytes susceptible to preferential recognition by LAK cells. Results are of a representative experiment repeated twice.

that host-derived heat-shock protein is not the species on infected monocytes that is recognized by LAK cells.

LAK Cells Contain More than One Killer Population. IL-2-induced lytic activity of LAK cells was associated with marked phenotypic changes, in particular, the expansion of subsets of cells with the appearance of large granular lymphocytes and the induction of certain surface antigens or expansion of subsets of cells bearing those antigens. Expressions of the CD56 antigen, the p55 chain of the IL-2 receptor, and MHC class II were induced.

The subset of $CD56^+$ cells in the peripheral blood includes the majority of IL-2-responsive cells and has been shown to contain the principle effectors in NK- and LAK-mediated lysis of tumor cells. Depletion of the 15–30% of $CD56^+$ cells from the LAK population removed cytotoxic activity against Daudi cells (over three CD56– LAK cells per target are required to achieve an efficiency of lysis mediated by ~0.75 undepleted LAK cells) and reduced killing of monocytes (Fig. 8). Killing of infected and uninfected targets was reduced by the same increment, therefore the extent of selective recognition of infected cells by $CD56^-$ LAK cells was not reduced. This suggests that depletion of $CD56^+$ cells reduced the lytic activity that recognized monocytes whether they were infected or not, but left intact the activity that selectively recognized infected cells. LAK cells that selectively recognized infected monocytes could also be generated by IL-2 activation of a CD56-depleted starting population (data not shown). Coculture of PBMC with mycobacterial antigen results in the generation of antigen-specific cytotoxic activity mediated by $CD4^+$ T lymphocytes (5), but depletion of $CD4^+$ cells failed to affect LAK-mediated lysis of either infected or uninfected targets, and positively selected $CD4^+$ LAK cells manifested no activity (data not shown). Both nonspecific cytotoxicity and selective recognition of infected cells were mediated by both $CD8^+$ and $CD8^-$ LAK cells (data not shown). Experiments are underway in the laboratory to further characterize the $CD56^-$ population that selectively recognizes infected monocytes.

Discussion

We have shown that human monocytes, chronically infected with BCG, are preferentially recognized and lysed by IL-2-activated LAK cells. This activity is mediated by syngeneic and allogeneic LAK cells, is not due to the presentation of mycobacterial antigen, and is associated with chronic infection, not the early stages immediately after phagocytosis. The recognition of target cells by antigen-independent, MHC-nonrestricted cytolytic cells like NK and LAK cells is poorly understood (6). Susceptible targets are bound and lysed but neither receptors nor ligands have been identified. In this system the nature of the determinants expressed by infected monocytes leading to enhanced recognition by LAK cells is also unclear. Chronic infection, or the presence of replicating, intracellular bacilli, may induced some change in host-encoded surface structures. Exposure of uninfected monocytes to heat shock does not enhance recognition by LAK cells, suggesting that the altered self is not a heat-shock protein as has been proposed in other systems (7). It has also been proposed that downregulation of MHC class I expression on certain tumor cell lines and virus-infected cells may confer susceptibility to lysis by NK cells (8). This does not appear to be important in this system since MHC class I expression is similar in both infected and uninfected cells. Current studies on the recognition of target cells by MHC-nonrestricted cytotoxic cells in other systems (9–11) may contribute to our understanding of the determinants of BCG-infected cells recognized by LAK cells.

Nonselective recognition of monocytes, like recognition of tumor cells, was mediated by $CD56^+$ LAK cells, but selective lysis of infected targets was mediated by $CD56^-$ LAK cells, suggesting that different cytotoxic populations

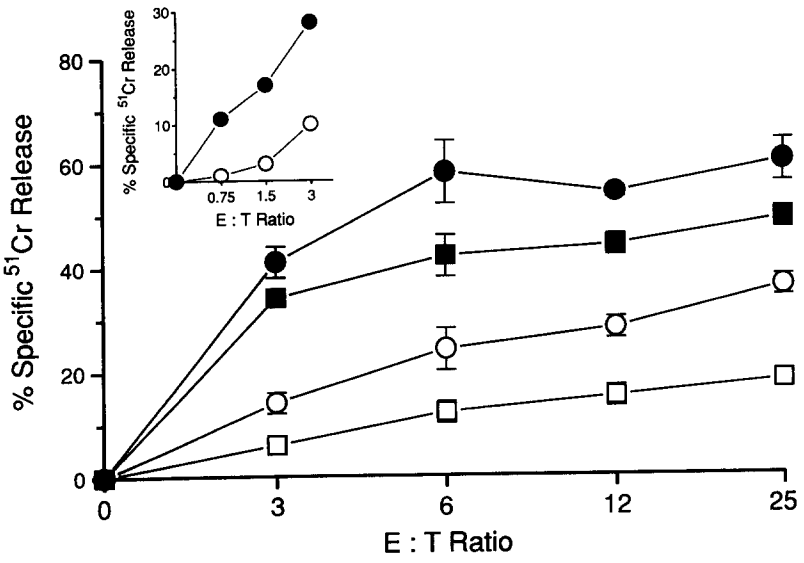

Figure 8. Selective and nonselective lysis is mediated by LAK cells with distinct phenotypes. 3–25 × 10^4 LAK cells (● and ○) or CD56-depleted LAK cells (■ and □) were incubated with infected (*filled symbols*) or uninfected (*open symbols*) targets. LAK cells contained 12–23% $CD56^+$ cells and $CD56^-$ LAK populations contained <2% $CD56^+$. Mock depletion (without primary antibody) did not alter LAK activity. Data are expressed as the mean ± SEM of two representative experiments. (*Inset*) 0.75–3 × 10^4 LAK cells (●) or CD56-depleted LAK cells (○) were incubated with Daudi cells in a parallel assay. Removal of $CD56^+$ LAK cells depleted Daudi killing and the nonselective activity against monocytes, without affecting selective recognition of infected cells.

are induced by coculture with IL-2. Further work is underway in the laboratory to characterize the $CD56^-$ LAK population that selectively recognizes infected cells. Most $CD56^-$ LAK are $CD3^+$. $CD4^+$ cells are inactive, and selective cytotoxicity does not segregate with CD8 expression, i.e., is mediated by both $CD8^+$ and $CD8^-$ populations. There is evidence that TCR γ/δ^+ T lymphocytes are directly responsive to IL-2 (12) and have an important role in immunity to mycobacterial infection (13). In this system TCR γ/δ^+ cells were expanded by coculture with IL-2, and selective recognition was reduced by depletion of TCR γ/δ^+ cells in some, but not all experiments. Therefore no consistent marker for the population that selectively recognizes and lyses infected monocytes, more specific than the absence of CD56, has been identified thus far.

Experiments performed in this laboratory have indicated that IL-2 has a central role in modulating innate immunity in chronic mycobacterial infection (14). Lepromatous leprosy patients, infected with *M. leprae*, are specifically anergic to *M. leprae* antigens. When recombinant IL-2 was administered intradermally to such patients, migration of lymphoid cells and selective destruction of infected phagocytes was observed in skin lesions distal to the site of IL-2 administration. This systemic effect occurred in the absence of the development of lymphocyte responsiveness to *M. leprae* antigens either in vitro or in vivo (15). This suggests that an antigen-independent, but selective, cytolytic mechanism was induced by cytokine administration. At the same time as the systemic destruction of infected macrophages was observed, an increase in peripheral blood LAK activity was detected (16). The data obtained in vitro reported here suggest that LAK cells induced in vivo by IL-2 therapy could have accounted for the selective destruction of infected macrophages observed in the patients.

The role of cell-mediated cytotoxicity in protection against mycobacterial infection is unclear. In vivo, after administration of IL-2 to anergic leprosy patients the selective destruction of parasitized macrophages, the migration of lymphoid cells into the skin, and the enhanced peripheral blood LAK activity were associated with a systemic reduction in the bacterial load (15). This suggests that LAK cells may have an important role in control of mycobacterial infection in patients. However, in vitro LAK-mediated cytolysis failed to inhibit intracellular BCG and resulted in the release of viable organisms. It is possible that in vivo LAK-mediated lysis of infected cells would release viable bacilli into the extracellular space. These would then be rephagocytosed by recently recruited monocytes. Inflammatory monocytes emigrating from the blood are better equipped with microbicidal machinery than aged tissue macrophages (17) and may be able to control or inhibit mycobacterial replication. Foci of infection, well characterized in mycobacterial disease (e.g., the pulmonary granuloma of tuberculosis), are sites of rapid trafficking and turnover of monocytes and lymphoid cells, lymphokine production, and tissue destruction (18). The understanding of protective immunity in mycobacterial infection may depend upon understanding the complex dynamics of this pecular microenvironment, in which LAK cells may be generated, and may play an important role.

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