DISSOCIATION OF OPSONIZED PARTICLE PHAGOCYTOSIS AND RESPIRATORY BURST ACTIVITY IN AN EPSTEIN-BARR VIRUS-INFECTED MYELOID CELL LINE

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ABSTRACT
A continuous tissue culture cell line (Karpas line 120), derived from a patient with acute myeloblastic leukemia, not only demonstrates myeloblastic morphology and in vitro expression of several myeloid-specific biochemical markers but also contains Epstein-Barr virus (EBV) nuclear antigen. The present studies demonstrate EBV-genome-specific DNA within the total cellular DNA by molecular hybridization, thus establishing the presence of stable viral genome integration. The cells demonstrate complex coordinated myeloid functions including ingestion, degranulation, and respiratory burst activity. Line 120 cells show a respiratory burst (superoxide and hydrogen peroxide generation and hexosemonophosphate shunt activity) in response to soluble (phorbol myristate acetate) and particulate (latex beads) stimuli, as do normal granulocytes. They ingest complement-opsonized particles (lipopolysaccharide-oil droplets, zymosan, and bacteria), and degranulate in response to them. However, unlike normal granulocytes, the line 120 cells do not demonstrate respiratory burst activity in response to these complement-opsonized particles. The dissociation between ingestion of complement-opsonized particles and activation of oxygen-dependent bactericidal activity severely impairs bacterial killing as compared with normal polymorphonuclear phagocytes.

Phagocytic cells respond to membrane contact with microorganisms by triggering a complex, coordinated sequence of activities including degranulation, ingestion, and a respiratory burst (1). The end result of this process is microbial killing, an essential part of host defence against infection by bacteria and fungi (1).

Human myeloid leukemia cell line 120 is a continuous tissue culture line derived from the peripheral blood of a patient with acute myeloblastic leukemia (16). The cells have the morphologic appearance of myeloblasts (16), contain alkaline phosphatase with "myeloid" substrate specificity (17) cytochemically inducible by butyric acid (13) or diffusion chamber incubation (14), have complement (C3) receptors (16), and generate superoxide anion (\(O_2^-\)) in response to the soluble stimulant phorbol myristate acetate (PMA).
MATERIALS AND METHODS

Labeled with tritium was prepared on a template of purified EBV cRNA-DNA hybridization was used for all surfaces in contact with cells. Calculations for ingestion and respiratory burst activity were corrected for the viable cell number. Plastic or siliconized glass was used for all surfaces in contact with cells.

120 cells were 50-75% viable by trypan blue exclusion. PMN were 98-99% viable, and line 120 cells were 1-5% eosinophils, and <1% mononuclear cells. All leukocytes (PMN) were isolated from the peripheral blood of one leukemic cell line with myeloid capabilities illustrates the difficulties of classifying such a line as "lymphoid" or "myeloid." Such classification involves morphological, biochemical, and functional characteristics that may not be entirely consistent with each other either in vitro (14) or in vivo (20).

**MATERIALS AND METHODS**

**Cells**

Karpas line 120 (3) was grown in 7% CO2 at 37°C in plastic flasks (Corning Glass Works, Science Products Div., Corning, N. Y.). Cells were passaged every 3 d in either (a) RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) with 10% heat-inactivated calf serum and 10% heat-inactivated calf serum (GIBCO) or (b) McCoy's 5a medium (Grand Island Biological Co., Grand Island, N. Y.). Cells were passaged every 3 d in either (a) RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) with 15% fetal calf serum and 10% heat-inactivated calf serum (GIBCO) or (b) McCoy's 5a medium (Grand Island Biological Co., Grand Island, N. Y.) . Cells were passaged every 3 d in either (a) RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) with 15% fetal calf serum and 10% heat-inactivated calf serum (GIBCO) or (b) McCoy's 5a medium (Grand Island Biological Co., Grand Island, N. Y.). Cells were passaged every 3 d in either (a) RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) with 15% fetal calf serum and 10% heat-inactivated calf serum (GIBCO) or (b) McCoy's 5a medium (Grand Island Biological Co., Grand Island, N. Y.). Cells were passaged every 3 d in either (a) RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) with 15% fetal calf serum and 10% heat-inactivated calf serum (GIBCO) or (b) McCoy's 5a medium (Grand Island Biological Co., Grand Island, N. Y.).

**cRNA-DNA Hybridization**

An EBV-specific complementary RNA (cRNA) probe radiolabeled with tritium was prepared on a template of purified EBV DNA (24). Hybridization of DNA extracted from 106 cells was conducted with an input of 105 cpm per membrane filter in duplicate for 22 h at 66°C under the same conditions described previously (24).

**Ingestion**

We measured ingestion of oil droplet particles (32, 33) and of 14C-labeled bacteria. In the former, a sonicated emulsion of diisodecylphthalate oil (Sigma Chemical Co., St. Louis, Mo.) containing oil red 0 dye (Sigma Chemical Co.) and E. coli lipopolysaccharide (Difco Laboratories, Detroit, Mich.) was opsonized by incubation in fresh or heat-inactivated (56°C for 1 h) human serum from the PMN donor for 30 min at 37°C, then incubated with cells (7 X 103 ml) for 5 min in Krebs-Ringer's phosphate buffer (KRP), pH 7.4, containing glucose 5 mM. Oil uptake was calculated from the absorbance (A) at 520 nm of dioxane extracts of washed cell pellets and the previously determined A520 of the emulsion. Complement-dependent ingestion was determined as the difference in oil uptake between opsonized (fresh serum) and nonopsonized (heat-inactivated) oil droplets.

14C-labeled bacteria were prepared by 48-h growth of E. coli strain 075:K1:O111 (kindly provided by Dr. Robert Daum) in minimal essential medium (Difco) containing 7 mM glucose, 500 μCi [14C]-glucose, and 50 μCi [14C]-leucine. Bacteria were washed, autoclaved, and then opsonized for 30 min at 37°C in fresh or heat-inactivated human serum. Cells (5 X 105/ml), bacteria (2.5 X 1010/ml) and serum (10%, fresh or heat inactivated) were incubated in KRP with glucose for 10 min at 37°C or 4°C, with shaking, then diluted in cold KRP, washed three times, and spun (200 g for 5 min), and the resuspended cell pellets were sampled for liquid scintillation counting. Ingestion was measured as the difference between counts per minute from cells incubated at 37°C and from cells at 4°C. There was no ingestion of bacteria incubated in heat-inactivated serum.

**Degranulation**

Washed zymosan (ICN K & K Laboratories Inc., Plainview, N. Y.) was opsonized in fresh or heat-inactivated human serum (37°C for 30 min), washed, and resuspended in KRP. Cells (7 X 105/ml) were then incubated for 30 min with opsonized zymosan (4 mg/ml) in KRP containing 5 μM cytochalasin B (Sigma Chemical Co.), placed on ice and centrifuged at 200 g for 10 min at 4°C. Whole cells and supernates were assayed for β-glucuronidase activity by the hydrolysis of p-nitrophenyl-β-D-glucuronide as previously described (22, 25). Complement-dependent degranulation was calculated as the difference in β-glucuronidase release by zymosan opsonized in fresh vs. heat-inactivated serum.

**Respiratory Burst Activity**

O2·− production was measured quantitatively by a previously described continuous spectrophotometric assay of superoxide dismutase-inhibitable cytochrome c reduction (6, 23). Assays were performed at 37°C in a double-beam spectrophotometer. Sample and reference cells both contained ferricytochrome c (50 nmol) (Sigma Chemical Co.), cells (2.5 X 105), and either PMA (1 μg Consolidated Midland Corp., Brewster, N. Y.) or opsonized zymosan (4 mg) in 1 ml total volume of KRP. When zymosan was the stimulus, cytochalasin B (5 μM) was added to increase O2·− detection by preventing internalization of phagocytic vacuo-
oles (38). The reference cell contained, in addition, superoxide dismutase (0.01 mg) (Sigma Chemical Co.). The rate of $O_2^-$ production was calculated by dividing the linear change in $A_{550}$ by the molar extinction coefficient for the reduction of ferricytochrome $c$ ($\Delta E = 21,000$) (21). The lag time for activation of $O_2^-$ generation was calculated as previously reported (6).

Previously described assays measured nitroblue tetrazolium (NBT; Sigma Chemical Co.) reduction in response to opsonized lipopolysaccharide-oil droplets (32) and PMA (23). Oil particles without red dye were prepared, opsonized, and incubated with cells as in the ingestion assay but with added NBT 0.04%, then dioxane-extracted in parallel with those for the ingestion assay. The amount of NBT reduced to formazan was calculated from the $A_{550}$ of dioxane extracts and the micromolar absorption coefficient of formazan (32).

The assay for HMPS activity measured the release of CO$_2$ from the first carbon of glucose (34). Cell suspensions (2.5 x 10$^6$ in 1 ml KRP buffer) were incubated with 2 mM glucose containing 0.1 $\mu$Ci/μmol of [1-$^3$H]glucose or [6-$^3$H]glucose (New England Nuclear, Boston, Mass.) and released $^3$HCO$_3^-$ was trapped in hyamine hydroxide (New England Nuclear) for scintillation counting. Glucose oxidation was examined both in resting cells and in cells stimulated by PMA (1 μg/ml), complement-opsonized zymosan (4 mg/ml), latex beads (10$^7$/ml), or complement-opsonized heat-killed bacteria (10$^7$/ml; E. coli 075 ab:Ko:Nm). HMPS activity was calculated as the difference between stimulated and resting CO$_2$ release from [1-$^3$H]glucose minus that from [6-$^3$H]glucose.

$O_2^-$ production was measured by a previously described continuous fluorometric assay of horseradish peroxidase-catalyzed scopoletin oxidation (30) calibrated by exogenous H$_2$O$_2$.

### NBT Slides

As previously described (23), cells (10$^7$/ml), NBT (1 mg/ml), and human serum albumin (20 mg/ml; Hyland Diagnostics Div., Travenol Laboratories, Inc., Costa Mesa, Calif.), and a stimulant in KRP were incubated for 20 min at 37°C in a shaker bath. The reaction was stopped with 10 cm$^3$ cold 0.154 M NaCl containing 0.001 M N-ethyl maleimide, centrifuged (10 min, 200 g), and washed twice more with the same solution. The final pellet was resuspended in 10 μl 5% albumin, smeared on glass slides, and counterstained with safranin. The amount of NBT reduced to formazan was calculated from the $A_{550}$ of dioxane extracts and the micromolar absorption coefficient of formazan (32).

The assay for HMPS activity measured the release of CO$_2$ from the first carbon of glucose (34). Cell suspensions (2.5 x 10$^6$ in 1 ml KRP buffer) were incubated with 2 mM glucose containing 0.1 $\mu$Ci/μmol of [1-$^3$H]glucose or [6-$^3$H]glucose (New England Nuclear, Boston, Mass.) and released $^3$HCO$_3^-$ was trapped in hyamine hydroxide (New England Nuclear) for scintillation counting. Glucose oxidation was examined both in resting cells and in cells stimulated by PMA (1 μg/ml), complement-opsonized zymosan (4 mg/ml), latex beads (10$^7$/ml), or complement-opsonized heat-killed bacteria (10$^7$/ml; E. coli 075 ab:Ko:Nm). HMPS activity was calculated as the difference between stimulated and resting CO$_2$ release from [1-$^3$H]glucose minus that from [6-$^3$H]glucose.

### Bacterial Killing

ATCC-25923 *Staphylococcus aureus* (kindly provided by Dr. Donald Goldmann) was opsonized in fresh human serum, sonicated for 15 s (setting 40 on a Sonifier, Branson Sonic Power Co., Div. of Branson Ultrasonics Corp., Plainview, N. Y.) and adjusted to OD$_{600}$ of 0.5 in PBS (=10$^6$ bacteria/ml). Mixtures of bacteria and cells, both at 5 x 10$^7$/ml in PBS containing 5 mM glucose and 10% fresh human serum, were sampled after 0, 30, and 90 min of shaking at 37°C. Samples were diluted first in distilled H$_2$O to lyse cells and then in PBS, plated, and incubated overnight: then the colonies were counted.

All reagents were obtained at the highest grade of purity available and used without further purification. All results are expressed as the mean ± SEM of triplicate determinations (except for oil droplet ingestion [duplicate] and bacterial killing [quadruplicate]). Each experiment was performed two to eight times.

### RESULTS

Infection of the 120 cell line by EBV was confirmed by hybridization of a previously characterized (24) $^3$H-labeled EBV cRNA probe to total cellular DNA. As shown in Table I, line 120 contained an estimated average of 61-74 EBV-genome equivalents per cell. The P3HR1 and Raji cell lines, established from African Burkitt's lymphomas, yielded 438 and 176 genome equivalents per cell, respectively. Negative control cells from

| DNA source | cRNA hybridized | cpm/50 μg DNA |
|------------|----------------|--------------|
| Line 120 A | 856            | 74           |
| B          | 800            | 70           |
| C          | 694            | 61           |
| P3HR-1 Burkitt's lymphoma cell line | 4,992 | 438 |
| Raji Burkitt's lymphoma cell line | 2,005 | 176 |
| HEp-2 human epithelial carcinoma cell line | 167 | <2 |
| 698 EBV-negative B cell lymphoma line | 95 | <2 |
| Calf thymus | 15            | <2           |

A $^3$H-cRNA probe specific to EBV DNA (24) was hybridized to DNA extracted from 10$^7$ cells of each designated type. Three determinations for line 120 and typical results from other cells are shown. The number of EBV genomes per cell was estimated on the basis of previously determined ratios of hybridized counts to known quantities of EBV DNA (24), except for the Raji line, in which the genome number was based on DNA-DNA reassociation kinetics. (Analyses carried out as in Pagano, J. S., and J. E. Shaw. 1979. Molecular probes and genome homology. In The Epstein-Barr Virus. M. A. Epstein and B. G. Achong, editors. Springer-Verlag, New York. 110-146).

- 167 cpm hybridized to HEp-2 DNA subtracted before genome determination.
the HEp-2 human epithelial carcinoma and 698 EBV-negative B lymphocyte cell lines and from calf thymus contained no detectable EBV DNA.

To evaluate the myeloid functional capacity of line 120 cells, we measured degranulation, ingestion, respiratory burst activity, and bacterial killing. Evaluation of degranulation in response to opsonized zymosan (Fig. 1) showed complement-dependent release of 23% of the total cellular enzyme activity from line 120 cells and 22% from PMN. The β-glucuronidase content of line 120 cells was 60% that of PMN.

Line 120 cells were also capable of ingestion of complement-opsonized particles. The complement-dependent uptake of 14C-labeled E. coli (Fig. 2) by line 120 cells was slightly over half that of PMN. They were also able to ingest complement-opsonized lipopolysaccharide-oil droplets (Fig. 3, left panel). However, as shown in the right panel of Fig. 3, line 120 cells failed to show any complement-dependent NBT reduction (i.e., <0.025 μg formazan/5 min per 10⁶ cells) in response to particles that the left panel shows them to be actively ingesting.

NBT reduction probably measures O₂⁻ production (3), a prime component of the respiratory burst of phagocytosis (1, 2). To further dissect the nature of the defect in the line 120 cell respiratory burst mechanism, we specifically examined generation of active oxygen species (O₂⁻ and H₂O₂) and HMPS activity in response to both complement-opsonized particles and PMA, a soluble stimulant (29). Fig. 4 shows that line 120 cells again failed to produce a respiratory burst in response to complement-opsonized particles (in this case, zymosan). They produced <0.01 nmol O₂⁻/min per 10⁶ cells, and showed no complement-dependent increase in HMPS activity over the resting state (Δ¹⁴CO₂ release < 10 cpm/30 min per 10⁶ cells). Line 120 cells also showed no HMPS response to complement-opsonized E. coli, which stimulated PMN to release 1170 ± 110 cpm/30
FIGURE 4 Respiratory burst response to complement-opsonized zymosan. Line 120 cells (lines indicating no detectable response) and PMN (stippled bars) were assayed for $\text{O}_2^-$ production (left) and HMPS activity (right) upon addition of zymosan. Results represent the difference in response to zymosan-opsonized in fresh vs. heat-inactivated serum. HMPS activity was also corrected for base-line $^{14}\text{CO}_2$ release (10 cpm/30 min per $10^6$ cells). Bar heights and error lines represent the mean ± SEM of triplicate determinations.

FIGURE 5 Respiratory burst response to PMA. Line 120 cells (clear bars) and PMN (stippled) were assayed for $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ production (both expressed as nanomoles per minute per $10^6$ cells) and for HMPS activity (increase in $^{14}\text{CO}_2$ release above resting cell levels) upon addition of PMA. Bar heights and error lines represent mean ± SEM of triplicate determinations.

related to the particulate nature of the complement-opsonized stimulants, the cells were exposed to unopsonized latex beads and to immunoglobulin-opsonized bacteria for the preparation of NBT slides. Line 120 cells both ingested latex beads (mean 4.1/cell, compared with PMN, 15/cell) and produced a respiratory burst in response to them. They reduced NBT (Fig. 6d) and activated the HMPS; $^{14}\text{CO}_2$ release above the resting level was 63 ± 7 cpm/30 min per $10^6$ cells (PMN, 734 ± 120). Line 120 cells showed no response to immunoglobulin-opsonized *Pseudomonas* or *Staphylococcus*. This finding is consistent with their previously demonstrated lack of Fc receptors (16).

The summation of the phagocytic functions described so far—degranulation, ingestion, and respiratory burst activity—is the killing of microorganisms. Fig. 7 shows that line 120 cells incubated with complement-opsonized *Staphylococcus aureus* show no significant bactericidal activity at 30 min and only a small amount of killing at 90 min incubation. PMN killed 90% and >99% of the *Staphylococci*, respectively, at those time points.

DISCUSSION

Previous studies have shown that cell line 120 contains the EBV nuclear antigen (a marker for B lymphoid cells [26]), yet expresses several myeloid cell-specific gene products and exhibits myeloblastic morphology (13, 14, 16, 17). The present demonstration of EBV-genome-specific DNA in these cells confirms that they have been infected with EBV, a property generally considered to be restricted to B lymphocyte cell lines (26). The
estimated number of EBV genomes per cell in line 120 is similar to that found in cell lines such as Raji that have the EBV genome incorporated into the cellular DNA and produce EBV nuclear antigen, but do not contain replicating virus. Much higher numbers of genomes can be demonstrated in EBV producer cell lines such as P3HR1 (26).

Despite the presence of this B lymphocyte marker, the line 120 cells demonstrate capabilities for several complex coordinated myeloid functions that probably represent many independent gene products. The cells generate $O_2^{-}$ and $H_2O_2$, increase HMPS activity with stimulation, ingest particles, and degranulate. Thus the cells exhibit a mixture of myeloid and lymphoid characteristics. The 120 cell line may derive from a common progenitor stem cell with the potential for myeloid and lymphoid differentiation. Evidence for a bi-potential stem cell exists in the finding of exclusive expression of a single G-6-PD isoenzyme in some (probably B) lymphoid and all myeloid cells of a G-6-PD heterozygote with chronic myelocytic leukemia (10). Alternatively, the cells may represent another example of the capacity of malignant cells to reverse repression of genes normally not expressed in the cell of origin. A closely related
analogy is the hairy cell leukemia leukocyte, which contains EBV genome and nuclear antigen but also exhibits monocytoid characteristics (20). Tumors and leukemic cells have been reported to express even more ontogenetically distant genes, such as parathyroid hormone secretion (25, 27).

The ratio of O$_2^-$ to H$_2$O$_2$ production differs in line 120 cells and PMN (Fig. 5). In the latter, the high ratio suggests that H$_2$O$_2$ is produced as a product of O$_2^-$ dismutation. In fact, addition of exogenous superoxide dismutase shifts the ratio to the theoretical 2:1 predicted by the reaction O$_2^-$ + O$_2^-$ → H$_2$O$_2$ + O$_2$ (6). The ratio observed in line 120 cells, slightly <1, could reflect decreased detection of O$_2^-$ or a shift in the oxidase system from one-electron reduction of O$_2$ (to O$_2^-$) to two-electron reduction (to peroxide). The ability of oxidase systems to vary the number of electrons transferred has been well-studied in xanthine oxidase (11) and suggested in human granulocytes ingesting latex particles (35).

The pattern of response of line 120 cells to different stimuli reveals a dissociation of the recognition and phagocytosis of complement-opsonized particles from the activation of the respiratory burst in these cells. The line 120 cells are capable of degranulation and ingestion responses to complement-opsonized oil droplets, E. coli, and zymosan. These activities are complement-dependent, having been calculated as the response to particles incubated in fresh serum minus the usually negligible response to the same particles opsonized in heat-inactivated serum. However, the line 120 cells do not activate the respiratory burst in response to these same complement-opsonized particles, even while ingesting them. They do not lack the enzymes necessary for the burst: they generate O$_2^-$ and H$_2$O$_2$ and increase HMPS activity in response to the soluble stimulant PMA. Nor do they incapable of response to a particle: latex beads, which require no opsonization and probably utilize a different surface receptor, stimulate NBT reduction and HMPS activation. Thus the line 120 cell complement receptor (16) is functionally intact and connected to the cytoskeleton, but its connection to the otherwise intact respiratory burst mechanism is blocked or absent.

The observed defect helps to elucidate several aspects of phagocyte physiology. The dissociation between complement and PMA stimulation of the respiratory burst suggests that the receptors or modes of activation for the two stimuli are separate. Furthermore, the finding of intact ingestion and degranulation but a defective metabolic response in line 120 cells exposed to complement-opsonized particles indicates that the membrane changes occurring in the former processes are different from those involved in the activation of the membrane-bound (9) O$_2^-$ generating system. In chronic granulomatous disease phagocytes, ingestion occurs normally without a respiratory burst response (8) but these cells, unlike line 120, fail to produce O$_2^-$, regardless of the stimulus. They probably lack the necessary oxidase or its activating system entirely, whereas, in line 120, both the oxidase and activating system are present but there is a dissociation between complement-mediated ingestion and the activation of the respiratory burst.

The functional consequence of this dissociation is the observed defect in bacterial killing. Indeed, the cells have bactericidal ability only to the minimal extent seen in chronic granulomatous disease leukocytes (28). The relatively slower ingestion rate of the line 120 cells (approximately one-third

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1 Weinbaum, R. L., and G. L. Mandell, University of Virginia. Personal communication.
that of PMN) probably contributes to the bactericidal defect but does not seem sufficient to explain the virtual absence of phagolysosomal killing by these cells. We have previously shown (23) that HL-60 cells, a line that differentiates in vitro from granulocytes to PMN, kill staphylococci as well as PMN after only 6 d of culture in dimethylsulfoxide. At that point, their rate of ingestion is still 40% that of PMN but their respiratory burst mechanism has fully matured. Wang-Iverson et al. (36) have reported that PMN exposed to low doses of PMA (20 ng/ml) have a 40-50% diminution in their ingestion capacity but normal killing at low to moderate bacteria to cell ratios (1:1 and 10:1) such as those used in the present study.

The very low level of bactericidal activity observed may derive from other, perhaps granule-associated, killing mechanisms or a very small amount of oxidative activity undetectable by other methods.

Recently, Harvath and Andersen described a patient having similarly defective leukocytes and repeated infections in a condition typical of chronic granulomatous disease (15). Complement-opsonized particles did not stimulate respiratory burst activity, but responses to soluble stimuli were normal in his granulocytes. Ingestion and degranulation in response to the particles appeared normal but bactericidal activity was depressed. Weening et al. (37) have described two siblings with a different triggering defect, also resulting in recurrent infections. The dissociation between opsonized particle phagocytosis and respiratory burst activity, now described both in a tissue culture cell line and a clinical setting, increases the number of possible defects that can lead to chronic granulomatous disease.

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