Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 454–458, January 1987
Cell Biology

Cytoplasts made from human blood polymorphonuclear leukocytes with or without heat: Preservation of both motile function and respiratory burst oxidase activity

(cytokineplasts/chemotaxis/phagocytosis/superoxide)

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Communicated by Aaron B. Lerner, October 1, 1986

ABSTRACT **Anucleate fragments (cytoplasts) from polymorphonuclear leukocytes (PMN) are simplified systems that can be used to elucidate specific pathways by which cell function is altered. PMN cytoplasts in current use are defective either in activatable respiratory burst oxidase activity or in motile function. By centrifugation of PMN on discontinuous gradients of Ficoll without cytochalasin B, we have created granule-poor cytoplasts in which both these capacities are preserved. Specifically, they generate superoxide anion (O_2^-) and reduce nitroblue tetrazolium dye on appropriate stimulation; they respond chemotactically to erythrocytes destroyed by laser microirradiation or to the specific chemoattractants fMet-Leu-Phe (10 nM) and C5a (zymosan-activated serum); and they ingest and kill staphylococci. We can improve the yield of these fragments progressively by preheating (45°C) the cells in suspension for increasing periods of time, but those treatments are attended by a decreasing percentage of cytoplasts with activatable oxidase activity, and a progressive inability of the cytoplasts to ingest and to kill staphylococci. These easily made and multipotent cytoplasts readily lend themselves to studies of PMN physiology.**

Two distinct granule-poor cytoplasts from human blood polymorphonuclear leukocytes (PMN) are currently being used to study functional pathways in the parent cell. Cytokineplasts (CKP; "kine," to emphasize their motile capacities), induced by the brief application of heat to adherent PMN, are former protopods (leading fronts, lamellipodia) that have become uncoupled from the main body of the cell. They retain motile functions of the parent PMN but lack respiratory burst oxidase activity (1–5). In contrast, cytochalasin B (CB) cytoplasts (neutrophil cytoplasts, neutroplasts), produced by the high-speed centrifugation of CB-treated PMN in discontinuous Ficoll gradients (6), have activatable oxidase activity (4, 6, 7) but defective motile function (4–6).

After heating cells in suspension instead of on surfaces (in an effort to produce CKP in bulk), we found that centrifugation on discontinuous Ficoll gradients without CB would release cytoplasts from PMN that had been preheated for periods that were suboptimal for CKP formation on surfaces. Yields increased with increasing periods of preheating. Depending on the time of preheating, these fragments retained both activatable oxidase activity and motile function. Moreover, cytoplasts with similar properties were released from control PMN that had been centrifuged without preheating.

MATERIALS AND METHODS

Preparation of Human Blood Leukocytes. As described (8), heparinized venous blood (250–300 ml) from normal volunteers was sedimented in dextran, and the leukocyte-rich supernatant (60–85% PMN) was sedimented and washed in modified heparinized Krebs–Ringer phosphate buffer (pH 7.4). The cells were osmotically shocked (to lyse erythrocytes), restored to isotonicity, washed once more in buffer, and counted (yield, generally 5×10^8 to 1×10^9 PMN).

Aliquots (5×10^7 to 1×10^8) of cells to be heated were sedimented (5 sec) in 1.7-ml plastic microcentrifuge tubes (Eppendorf centrifuge 5412, Brinkmann), the supernatants were removed, the tubes were plunged into a water bath at 45°C, and the cells were immediately resuspended in buffer preheated to 45°C. A temperature probe confirmed that the cells remained at 44.5°C–45°C. After prescribed intervals (3–14 min) the tubes were removed from heat, the cells were sedimented, and the button was resuspended in 12.5% (wt/vol) Ficoll preheated to 37°C for immediate use. In some experiments, PMN were pretreated with 10 μM colchicine for 30 min at 37°C to disrupt microtubules.

Preparation of Cytoplasts. Cells, preheated or not, suspended in 12.5% warm Ficoll (see above), were layered on a discontinuous gradient of 16% Ficoll and 25% Ficoll, and cytoplasts were prepared by centrifugation for 30 min at $81{,}000 \times g$ in a rotor prewarmed to 37°C, according to the method of Roos *et al.* (6), but without CB. In experiments with colchicine, the gradient also contained the drug at 10 μM. The band of cytoplasts was harvested from the 12.5%/16% interface, diluted in buffer, sedimented, and washed in buffer five more times in the microcentrifuge. For tests of function, a purity of ≥97% was required.

Chemotaxis. A chemotactic gradient lasting several minutes was produced in sealed microscopic slides by destruction of erythrocytes by a ruby laser (6943 Å, 3 J, 500 μsec, 5-μm beam diameter), as described (2, 9). The response of individual fragments was followed in phase-contrast microscopy in a Zeiss Photomicroscope 1 and recorded in serial photographs on Kodak 35-mm Panatomic-X film. For response to specific chemotactic factors, we used a Zigmond chamber (2, 10), in which fragments migrate across a bridge between two parallel trenches, one of which contains the putative chemoattractant. In chambers warmed to 37°C (in hot air), we followed the locomotion of fragments in time-lapse phase-contrast videomicroscopy (Hamamatsu TV C1000 camera, Sony KCA-60K tape).

Electron Microscopy. Cells or fragments were fixed in suspension at 37°C with an equal volume of 2% (wt/vol) glutaraldehyde in buffer, and postfixed in 1% OsO_4. Ultrathin

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Abbreviations: PMN, polymorphonuclear leukocytes; CKP, cytokineplasts; CB, cytochalasin B; PMA, phorbol 12-myristate 13-acetate; NBT, nitroblue tetrazolium.

sections were stained with uranyl acetate and lead citrate and examined in a Zeiss EM-10CA transmission electron microscope at 60 kV. Sections (1–3 μm thick) were stained with Azure 1 and 2.

Phagocytosis and Killing of Staphylococci. The phagocytic system was essentially as described (8). Briefly, 0.5–3 × 10^7 PMN or cytoplasts in 1 ml of buffer were added to 1.3 ml of additional buffer and 0.3 ml of autologous serum and shaken in 25-ml Ehrlenmeyer flasks at 37°C in an Eberbach Incubator Model 6250 (Eberbach Corp., Ann Arbor, MN), 100 reciprocations per min. After ≈5 min, 0.2 ml of an overnight culture of *Staphylococcus aureus* strain 502A was added (zero time) at *Staphylococcus*/PMN (or of *Staphylococcus*/cytoplast) ratios of 10–35:1, and the shaking incubation continued. After 20 min, and again after 60 min of incubation, duplicate samples were removed to homogenization tubes, and the cells or fragments were disrupted by Teflon glass homogenization (1 min, ice-water bath) either directly or after separation of samples into supernatant and cell (or fragment)-associated fractions. Remaining live bacteria were enumerated by duplicate quantitative plating in agar and compared to the inoculum. Bacterial controls were also enumerated after incubation, both without cells or fragments (serum controls) and with cells or fragments that had been disrupted before incubation by five cycles of freezing (in ethanol/dry ice) and thawing. Other samples at 60 min were smeared, stained (Wright's stain), and 200 consecutive cells or fragments were examined for numbers of associated bacteria.

Respiratory Burst Oxidase Activity. Among individual PMN or cytoplasts, activatable oxidase activity was measured by the percentage of 200 consecutive cells or fragments able to reduce nitroblue tetrazolium (NBT) dye to blue formazan precipitate on endotoxin-coated slides (normal PMN, >90%) (11). For cells or fragments in suspension, the generation of superoxide (O_2^-) anion by leukocytes or cytoplasts was measured on stimulation with phorbol 12-myristate 13-acetate (PMA) (100 ng/ml), as the superoxide dismutase-inhibitable reduction of ferricytochrome *c*, as described (12).

Reagents. fMet-Leu-Phe (Sigma), PMA (Sigma), and CB (Aldrich) were prepared as stock solutions in dimethyl sulfoxide and diluted in buffer before use. Colchicine (Sigma) was freshly prepared in aqueous medium.

RESULTS

Effect of Preheating on Yield of Cytoplasts. Yields varied a great deal but tended to be a direct function of the time of preheating of the parent PMN. For example, in several experiments with PMN preheated for 0, 3, 6, 7, 8, or 9 min, the percentage yields (mean ± SD) of cytoplasts were 22 ± 16, 32 ± 29, 53 ± 23, 56 ± 27, 67 ± 30, and 71 ± 34, respectively (n = 16, 16, 5, 11, 5, and 5, respectively). After 13 min of preheating, yields decreased somewhat. Thus, preheating facilitated the pulling apart of the cells on the discontinuous gradient, and a certain percentage of centrifuged cells released cytoplasts without having been preheated.

Colchicine pretreatment did not affect yields of nonpreheated cytoplasts. In six additional paired experiments, yields (mean + SD) were the same with and without the drug: 18% ± 7%.

Stained and viewed in the light microscope, these anucleate fragments contained few or no granules. An electron micrograph of nonpreheated fragments is shown in Fig. 1. They had rare granules and variable amounts of membranous material. Fragments from preheated PMN had a similar appearance.

Cells in suspension appear to be more resistant to heat than cells adherent to surfaces. On surfaces, 9 min (at 45°C) is the optimal heating time for PMN to form CKP in high yield (2). In contrast, when cells were heated in suspension and then placed on surfaces (instead of being centrifuged), it took 13–14 min of preheating for CKP to form, and yields were poor. The combination of briefer heating with centrifugation therefore permitted separation of cytoplasts from a population of PMN that did not form them without centrifugation.

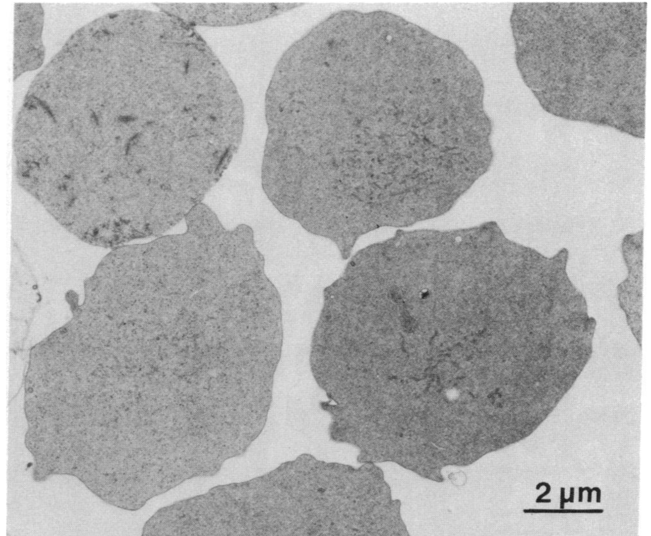

FIG. 1. Cytoplasts produced by centrifugation of PMN on discontinuous gradients of Ficoll, without preheating and without CB, are seen in the transmission electron microscope. Note the general lack of granules and variable amounts of membranous material. In light microscopy, stained whole fragments also had few or no granules. (×5300.)

Motile Function of Cytoplasts. Like CKP, the cytoplasts could adhere to glass, spread, and exhibit membrane movement under direct microscopic observation. For studies of locomotion and chemotaxis, we concentrated on fragments from cells that had been preheated for only 3 min, or not at all (henceforth referred to as 3-min or 0-min cytoplasts or fragments). Fig. 2 shows the chemotactic response of such fragments to erythrocytes destroyed by laser microirradiation. Note that virtually all the cytoplasts are responsive. Both 0-min and 3-min cytoplasts also responded chemotactically in gradients of the specific chemoattractants fMet-Leu-Phe (Fig. 3) and C5a (zymosan-activated serum).

Phagocytosis, Killing of Staphylococci, and Metabolic Activity of Cytoplasts. Some degree of ingestion of staphylococci was preserved among cytoplasts created from PMN preheated for as long as 13 min (Fig. 4). However, the ability of cytoplasts to kill the bacteria decreased as a direct function of the time of preheating (Fig. 5). This killing capacity correlated directly with the percentage of individual cytoplasts that were able to reduce NBT dye to formazan—i.e., that retained evidence of respiratory burst oxidase activity (Fig. 5). As another measure of oxidase activity—this time of the suspended fragments as a whole—we examined the generation of superoxide anion (O_2^-) by the cytoplasts on stimulation by PMA (Fig. 6*A*), and compared it to that of CKP, CB cytoplasts, and intact parent cells (Fig. 6*B*). Like NBT reduction, $O_2^{-\cdot}$ generation was an inverse function of the time of preheating. $O_2^{-\cdot}$ generation by 0-min and 3-min cytoplasts was similar to that of CB cytoplasts.

The progressive increase in survival of staphylococci shown in Fig. 5 is attributable both to cytoplasts taking up fewer bacteria according to the time of preheating of their parent PMN, and to a defect in killing those that they do take up. In three experiments in which *Staphylococcus*/PMN or fragment ratios were 32:1, 25:1, and 12:1, the similar per-

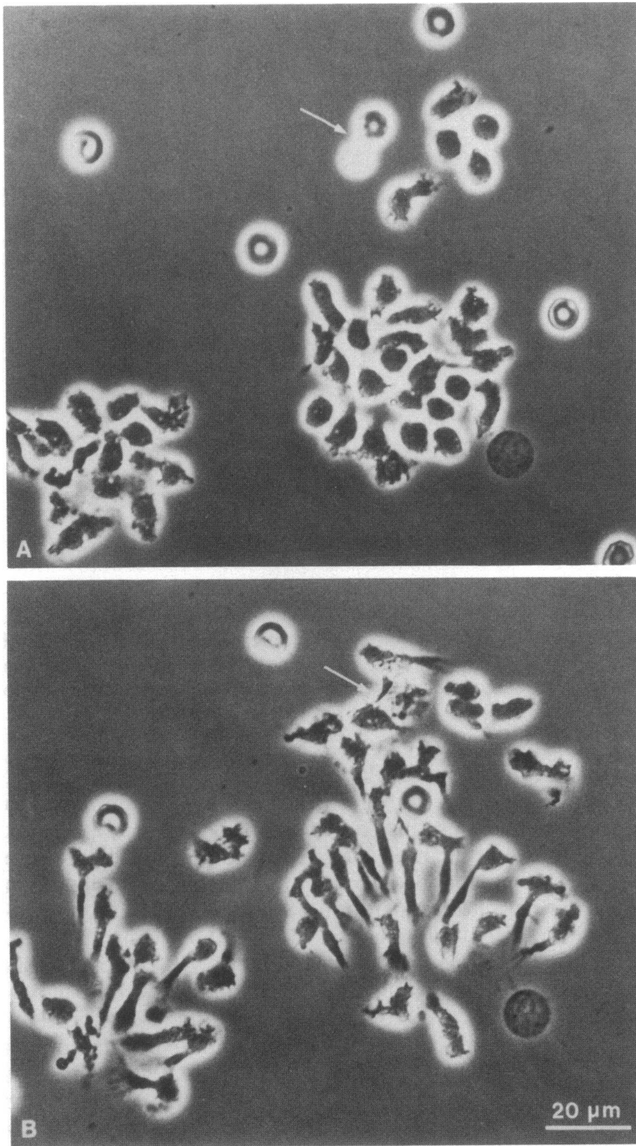

FIG. 2. Chemotactic behavior of cytoplasts created from PMN that had been preheated in suspension (45°C for 3 min) and then subjected to discontinuous gradient centrifugation. (*A*) Field containing two major nests of cytoplasts. Arrow points to two erythrocytes (the lower one is crenated) before their destruction by laser microirradiation, which creates a chemotactic gradient. (*B*) Same field as in *A*, minutes later. Some cytoplasts have reached the destroyed erythrocytes (arrow). Many others in this particular preparation are especially adherent to glass; they repeatedly stretch toward the target but are anchored to substratum by attenuated "tails." Note that virtually all the cytoplasts are responsive. Phase contrast microscopy. (×575.)

centage results were combined in Fig. 7. The 0-min cytoplasts were intermediate in their ability to remove bacteria from the medium (supernatant fraction) between the intact parent PMN (the most efficient) and 7-min cytoplasts. Despite decreased uptake, 7-min cytoplasts retained more live bacteria (fragment-associated fraction) than did PMN. For example, at the ratio of 32:1, we recovered ≈5 live staphylococci per 7-min cytoplast, versus ≈3 per 0-min cytoplast and <1 per intact cell. Cytoplasts disrupted by freezing and thawing (F/T) were unable to take up or kill bacteria.

The order of efficiency of uptake—PMN > 0-min cytoplasts > 7-min cytoplasts—was confirmed by actual counts of bacteria per cell or per fragment at 60 min of incubation. The distribution at 32:1 is shown in Fig. 8. At

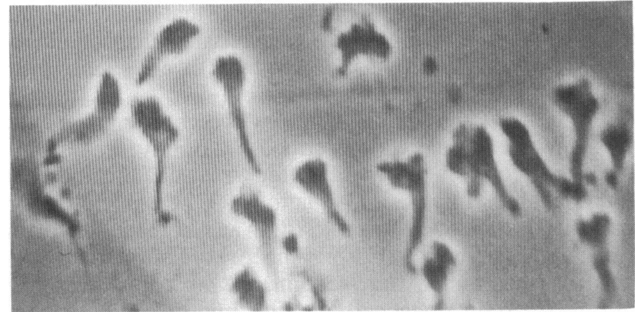

FIG. 3. Cytoplasts produced as described in Fig. 2 are seen in a Zigmond chamber, migrating toward a trench (top; not seen) containing the chemotactic peptide fMet-Leu-Phe (10 nM) in Hanks' balanced salt solution. They responded similarly to C5a (zymosan-activated serum). Cytoplasts derived from nonpreheated PMN also responded to both chemoattractants. Photographed from videotape. Phase contrast microscopy. (Approximately ×860.)

lesser challenges (25:1 and 12:1), the distribution of especially the 7-min cytoplasts skewed more to the left (not shown). Hence, the percentages of these cytoplasts with no bacteria at ratios of 32:1, 25:1, and 12:1 were 23%, 33%, and 46%, respectively; corresponding values for the more efficient parent PMN were 0%, 2%, and 8%.

DISCUSSION

Types of Cytoplasts. PMN can now be induced to release a number of varieties of granule-poor anucleate fragments (cytoplasts), each with its own uses for the study of leukocyte function. *(i) CKP*. The CKP, produced from PMN on surfaces, results from a heat-induced uncoupling of the hyaline microfilament-rich cortical cytoplasm (ectoplasm) from the nucleus and granuloplasm (endoplasm), possibly involving damage to centrosomal microtubule-organizing centers (2, 13). The membrane-bounded ectoplasm, massed

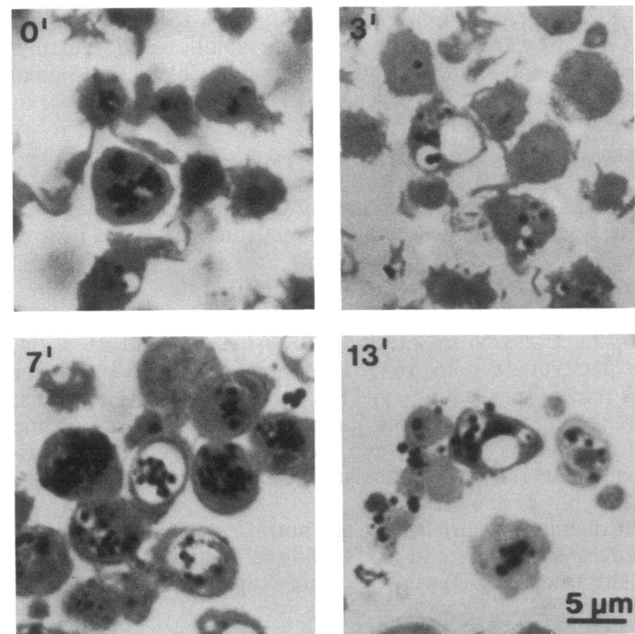

FIG. 4. Ingestion of staphylococci by cytoplasts created from PMN that had either not been preheated (0') or had been preheated (45°C) in suspension for 3, 7, or 13 min before discontinuous gradient centrifugation. In 1- to 3-μm sections of fixed sedimented cytoplasts, fragment-associated staphylococci are seen, sometimes with considerable vacuolization of the fragments. Stain, Azure 1 and 2; light microscopy. (×1600.)

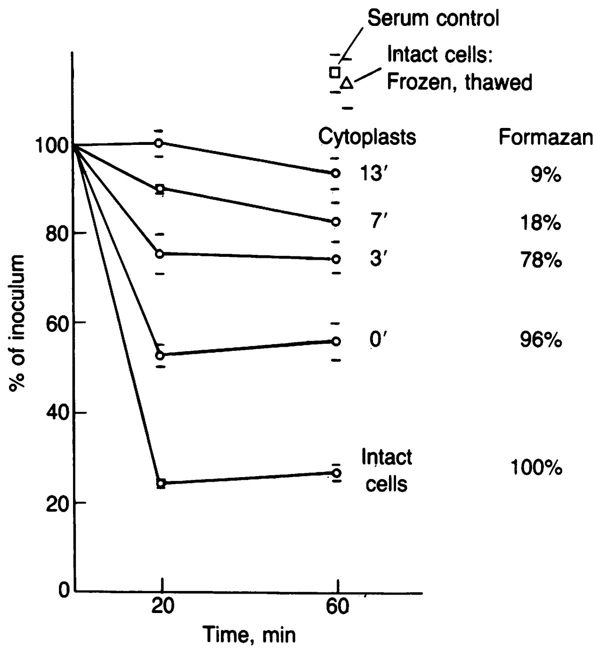

FIG. 5. The killing of staphylococci is shown by intact cells and by cytoplasts made as described in Fig. 4. The mean percentages of live staphylococci recovered from duplicate samples taken after 20 min of incubation, and again after 60 min, are indicated. Brackets indicate individual percentages. In general, survival of staphylococci varied inversely with the percentage of cytoplasts that were able to reduce NBT dye to formazan. The serum control contained medium and staphylococci, but no cells or fragments. Disrupted PMN (intact cells: frozen, thawed) did not kill staphylococci. PMN or fragments, 5×10^6 per flask; staphylococci/PMN or fragment ratio, 25:1.

as a protopod (leading front, lamellipodium), then crawls away from the residual cell body and detaches from it. The resulting CKP represents a (self)-purification of the cell's

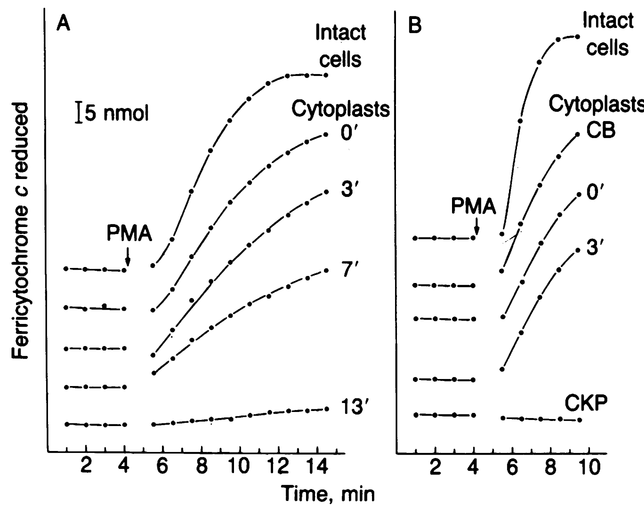

FIG. 6. Generation of superoxide anion by intact PMN and by various cytoplasts on stimulation with PMA. Superoxide production is measured at 550 nm as superoxide dismutase-inhibitable reduction of ferricytochrome c (nmol per 2.5×10^6 cells or fragments). Curves are vertically offset for clarity. (*A*) Cytoplasts prepared as described in Figs. 4 and 5. As with NBT reduction, superoxide generation varied inversely with the time of preheating of the parent PMN in suspension. Each plot is the mean of four runs. (*B*) Responsiveness of two of the cytoplasts is comparable to that of fragments made in CB. CKP, induced from PMN heated on surfaces (45°C, 9 min), are unresponsive. Cells or fragments per assay, 2.5×10^6; PMA, 100 ng/ml.

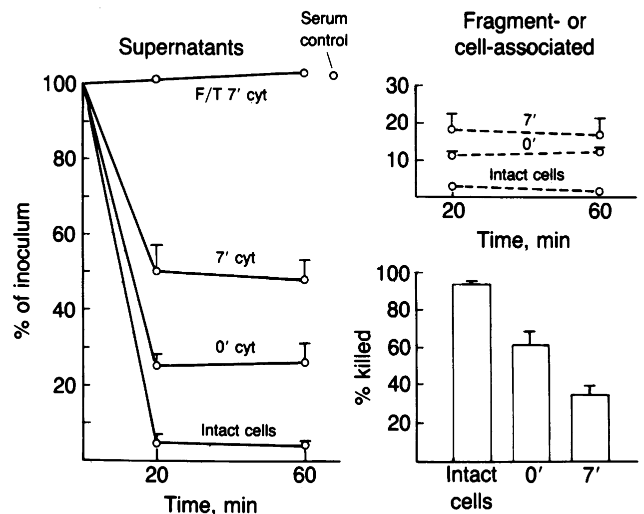

FIG. 7. Uptake and killing of staphylococci by intact cells and by cytoplasts (cyt) is shown where samples are divided into supernatant and cell (or fragment)-associated fractions. The design is as in Fig. 5, except that the results of three separate experiments have been combined; means + SEM are indicated. Cytoplasts from non-preheated PMN (0 min) removed bacteria from the medium (supernatant) more efficiently than did cytoplasts from PMN preheated for 7 min, but less so than did their larger and granule-rich parent cells. Despite decreased removal, cytoplasts appeared to kill fewer of the staphylococci that they did take up than did intact cells (i.e., more staphylococci were recoverable from the fragment fraction). The bars depicting % killed are derived from the line drawings at 60 min: 100 − (supernatant + fragment- or cell-associated fractions). Disrupted cytoplasts (F/T 7 min; two experiments) behaved like serum controls, which contained no cells or fragments. PMN or fragments, 3×10^7 per flask; staphylococci/PMN or fragment ratios for the three experiments, 12:1, 25:1, and 32:1.

motile machinery, with preservation of the sensing and transducing apparatus necessary for specific motile functions. It has been used to elucidate aspects of the parent PMN's motive force, function of centrosome and microtubules, longevity, mobilizable calcium reserves, and killing capacity (2–5, 13–15). However, a heat-induced extinction of respiratory burst oxidase activity prevents the CKP being useful for studies of oxidative function, except for the possible consequences of its absence (3, 15).

(ii) CB cytoplasts. For studies of oxidative activity, the CB cytoplast has been instructive. These fragments are pulled away from PMN on discontinuous gradients of Ficoll at a time when microfilaments are in CB-induced disarray (6).

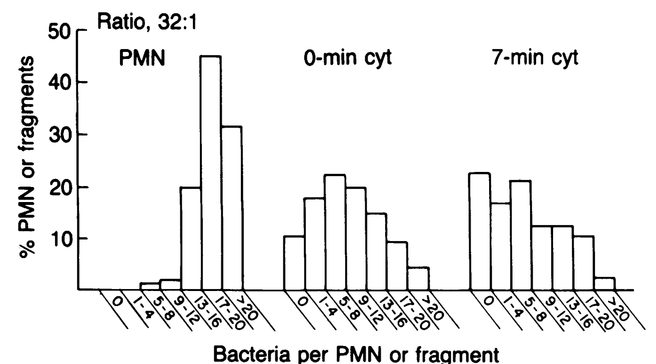

FIG. 8. Histogram showing distribution of bacteria per cytoplast (cyt) or per parent PMN in a single experiment. Staphylococci/PMN or fragment ratio, 32:1. Two hundred consecutive cells or fragments were examined after 60 min of incubation. As in the supernatant fraction in Fig. 7, the order of efficiency of uptake is PMN > 0-min cytoplasts > 7-min cytoplasts.

The resulting cytoplasts retain activatable oxidase activity, as well as such functions as fMet-Leu-Phe-induced aggregation, and adaptation of formyl peptide receptors, and they have been used to analyze the requirements for these functions (6, 7, 16). However, even though the preparative CB has been washed away, CB cytoplasts retain defective motile function (4–6), associated with an inability to polymerize actin normally when stimulated with fMet-Leu-Phe or with PMA (5).

(iii) New cytoplasts. Rather than a particular cytoplast, we are actually dealing with an array of motile granule-poor cytoplasts that retain activatable oxidase activity as an inverse function of the time of preheating (Figs. 5 and 6). Thus, the extinction of activatable oxidase activity by heat is dosage dependent. Although the progressive heat-induced killing defect for staphylococci (Fig. 5) is attributable in part to the combination of decreased oxidase activity and decreased efficiency of uptake (Figs. 7 and 8), we have not ruled out the possibility that heat can either inactivate bactericidal granular components or prevent their translocation to the plasma membrane during cytoplast formation. [Translocation of certain granular components has been demonstrated during the formation of CB cytoplasts (17).] Hence, granular elements might be contributing to killing of bacteria, even though intact granules are scarce.

Yield of cytoplasts appears to be a direct function of the time of preheating. This progression could indicate a progressive heat-dependent weakening of the normal coupling mechanism between cortical cytoplasm and granuloplasm, which the centrifugation then stresses to the breaking point. A requirement for such a formulation is that within a population of PMN there is a continuum in sensitivity of the coupling mechanism to heat. At one extreme are cells that form cytoplasts on centrifugation without having been preheated at all—i.e., the 0-min cytoplasts. Supporting this view is the observation by Bessis of the rare formation of CKP from unheated PMN on surfaces (an example recorded on film is described in ref. 2, p. 972).

Production of Cytoplasts by Centrifugation. In general, then, for the large majority of untreated PMN that cannot be pulled apart under these conditions of centrifugation, there are at least two ways to "soften them up" so that they can be. At first glance, both methods appear to attack the integrity of cytoskeletal elements: treatment with CB, by disrupting networks of microfilaments in the ectoplasm (cortical cytoplasm); treatment with heat, by interfering with the organization of microtubules (in the endoplasm, or granuloplasm) emanating from the centrosome. However, at least the effect of heat is likely to be more complicated than that, because treatment of cells with colchicine at a dosage that disrupts microtubules in PMN, combined with centrifugation, did not result in increased yields of cytoplasts.

Whatever the mechanism, heat, like CB, may be most useful in permitting the production of cytoplasts from cells that otherwise would not release them. However, both manipulations give rise to the same problem: alterations in function may be due to the enabling treatment rather than to the absence of a nucleus or other cellular elements. In the case of PMN, the 0-min cytoplasts appear to obviate this ambiguity. Hence, the differences in function between 7-min and 0-min fragments (Figs. 6–8) can fairly be ascribed to heat, and the relative motile disability of CB cytoplasts (4–6) can be ascribed to CB. Effects of the centrifugation itself on motility can be estimated by close comparison of 0-min cytoplasts with (uncentrifuged) CKP (2); so far, none is apparent. Because of their ease of production and multipotency, cytoplasts released from nonpreheated and non-CB-treated parent PMN may become the most useful of the available fragments.

We thank Professor Marcel Bessis, in whose laboratory the laser microirradiation was done; A. de Boisfleury Chevance, M. G. Breitenstein, and S. B. Cretella for expert technical assistance; and M. Livieri for preparation of the manuscript. This work was supported in part by grants from the U.S. Public Health Service (AM-10493, AM-07107) and by the Arthritis Foundation and its Connecticut Chapter.

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