# Cryopreservable Neutrophil Surrogates

## Stored Cytoplasts from Human Polymorphonuclear Leukocytes Retain Chemotactic, Phagocytic, and Microbicidal Function

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## Abstract

**Cryopreservation of polymorphonuclear leukocytes (PMN) has largely failed, probably because of their rich content of granular (lysosomal) enzymes. We have been developing granule-poor cytoplasts (anucleate fragments) from PMN which retain motile functions of the parent cell. The two types studied here were induced either by brief heating on surfaces (cytokineplasts) or by discontinuous gradient centrifugation (Ficoll) without heat or drugs (U-cytoplasts). Freshly made, these cytoplasts respond chemotactically to formyl peptide (fMet-Leu-Phe), and they take up and kill roughly half as many *Staphylococcus aureus* as their (larger, granular) parent PMN. Unlike their parent cells, after cryopreservation both cytoplasts remain chemotactic, and in matched experiments they take up and kill staphylococci with undiminished avidity. These findings are the first indications that PMN cytoplasts suitable for clinical use may be feasible.**

## Introduction

Cryopreservation of human blood polymorphonuclear leukocytes (PMN, neutrophils) is an old dream that has been found impractical; recovery after thawing has been low, and viability of cells has decreased rapidly at 37°C, probably because of post-thawing damage from granular enzymes (2–4). More recently, Voetman et al. (5) found that the majority of granule-poor cytoplasts (anucleate fragments) made from PMN in cytochalasin B are in fact cryopreservable, but the authors saw no clinical future for these fragments because they lack chemotactic responsiveness and their bactericidal activity is low.

We have been developing granule-poor cytoplasts from PMN which retain motile functions of the parent cell. Cytokineplasts (CKP),[1] produced from PMN on surfaces, result from a heat-induced uncoupling of the hyaline microfilament-rich cortical cytoplasm (ectoplasm) from the nucleus and granuloplasm (endoplasm) (6, 7). The membrane-bounded ectoplasm, massed 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 motile machinery, with preservation of the sensing and transducing apparatus necessary for specific motile functions (6–11). Surprisingly, CKP also retain considerable microbicidal capacity (11) despite having few or no granules and a heat-induced extinction of respiratory burst oxidase activity.

In an effort to find the proper conditions of preheating to produce cytoplasts by centrifugation of suspended PMN in discontinuous gradients of Ficoll (without cytochalasin B), we created an array of motile, granule-poor cytoplasts that retain activatable oxidase activity as an inverse function of the time of preheating (12). It became apparent that cytoplasts could be made in these gradients even from PMN that had not been preheated at all, and were therefore called "O-min" cytoplasts (12). These latter fragments combine retention of activatable oxidase activity, as seen in cytoplasts made in cytochalasin B, with retention of motile and microbicidal properties, as seen in CKP. Here we have renamed them U-cytoplasts (U-CYT), the "U" signifying that they are unpretreated, either by heat or by cytochalasin B. This study examines the cryopreservability of U-CYT and CKP.

## Methods

*Preparation of human blood leukocytes.* Essentially as described previously (13), heparinized venous blood (350 ml) from normal volunteers was sedimented in 2 vol of 3% dextran, and the leukocyte-rich supernatant (60–85% PMN) was sedimented and washed in HBSS (Gibco, Grand Island, NY). The cells were osmotically shocked (to lyse red cells), restored to isotonicity, washed once more in buffer, and counted (yield: generally about 8–13 × $10^8$ PMN). Aliquots were set aside at room temperature for same-day use, or cryopreserved (below), and the remainder used to produce cytoplasts.

*Preparation of U-CYT.* As described in detail previously for the preparation of what were then called 0-min cytoplasts (12), aliquots (about 5 × $10^8$) of purified leukocytes were suspended in 12.5% (wt/vol) warm Ficoll and layered on a discontinuous gradient of 16% Ficoll and 25% Ficoll, and cytoplasts were prepared by centrifugation for 30 min at 81,000 *g* in a rotor prewarmed to 37°C, according to the method of Roos et al. (14), but without cytochalasin B. The band of cytoplasts was harvested from the 12.5%/16% interface, diluted in buffer, sedimented, washed in buffer five more times by microcentrifugation, and counted (yield: 10–40%). Aliquots were set aside at room temperature for same-day use, or cryopreserved (below).

*Preparation of CKP.* As described in detail previously (7, 9), aliquots (about 7 × $10^8$ purified leukocytes in buffer with 20% fetal calf

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1. *Abbreviations used in this paper:* CKP, cytokineplast(s); NBT, nitroblue tetrazolium; Staph, *Staphylococcus aureus* strain 502A; U-CYT, unpretreated cytoplast(s).

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serum were distributed among glass plates, allowed to adhere at 37°C, heated for 9 min on glass whose surface registered 45°C, and returned to 37°C to allow the maximum number of motile fragments to move away from PMN bodies and detach (30–45 min) (7). Mononuclear cells do not produce fragments under these conditions, but virtually all the PMN do (7, 15). Fragments and residual cell bodies were harvested, pelleted, resuspended in warm 12.5% Ficoll, purified on discontinuous gradients as above, and counted (yield: 10–20%). Again, aliquots were set aside fresh, or cryopreserved.

*Conditions of freezing and thawing.* Standard methods were employed (5). PMN, CKP, or U-CYT, each at about $1 \times 10^7$ in 2-ml polypropylene tubes, were sedimented and made up first in 0.5 ml of HBSS containing 10% FCS, 4°C, then in an additional 0.5 ml HBSS-FCS and 20% DMSO, 4°C. The tubes surrounded by styrofoam were placed in a −80°C freezer for one to 6 d. For thawing, tubes were shaken by hand at 37°C until their contents had thawed completely. The cells or cytoplasts were washed in 14 ml of HBSS with 10% FCS, and twice more in 2 ml of their test media, and set aside at room temperature until use.

*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%) (16). For cells or fragments in suspension, the generation of superoxide ($O_2^-$) anion by leukocytes or cytoplasts was measured on stimulation with PMA, 100 ng/ml, or with fMet-Leu-Phe, 100 nM, as the superoxide dismutase–inhibitable reduction of ferricytochrome *c* (17).

*Chemotaxis.* For response to the chemotactic peptide fMet-Leu-Phe (Sigma Chemical Co., St. Louis, MO) we used Zigmond chambers (7, 18), 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).

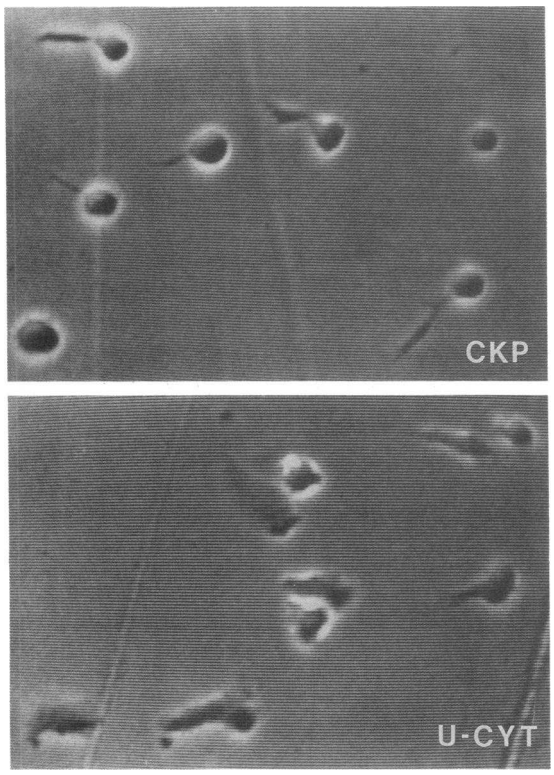


*Figure 1.* Chemotactic responsiveness of cytoplasts after cryopreservation. Thawed CKP and U-CYT are seen in Zigmond chambers, migrating toward trenches (*right; not seen*) containing the chemotactic peptide fMet-Leu-Phe (10 nM). Photographed from videotape. Approximately ×500.

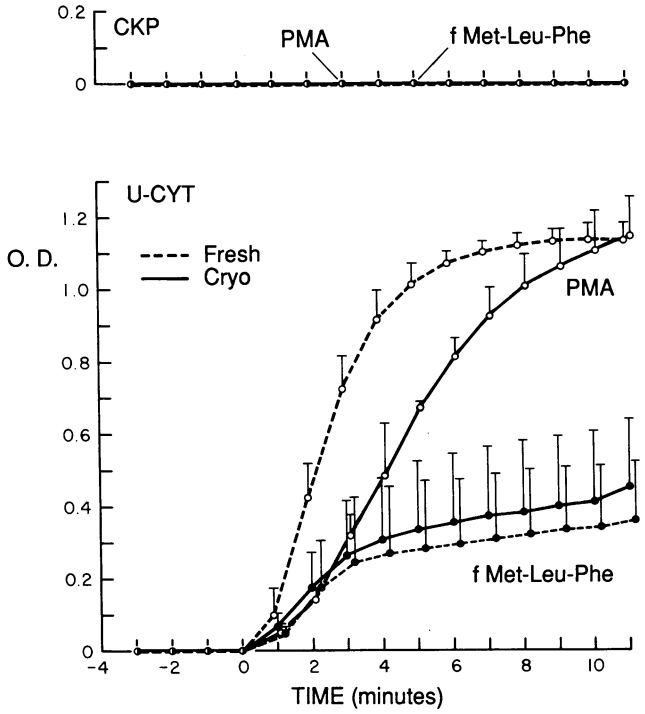


*Figure 2.* Generation of superoxide anion by cytoplasts before and after cryopreservation. Superoxide production is measured at 550 nm as superoxide dismutase–inhibitable reduction of ferricytochrome *c*. Fragments received PMA or fMet-Leu-Phe at zero time. (*U-CYT*) The mean + SEM is seen for three experiments. For each agonist, the amount of anion generated by matched aliquots of fresh and cryopreserved (*Cryo*) fragements are similar by 10 min, despite a brief lag period among thawed fragments given PMA. (*CKP*) The lack of activatable oxidase in fresh fragments persists after cryopreservation. Fragments per assay, $5 \times 10^6$; PMA, 100 ng/ml; fMet-Leu-Phe, 100 nM.

*Bacteria. Staphylococcus aureus* strain 502A (Staph) was shaken overnight in beef heart infusion broth at 37°C and centrifuged, and the bacteria were suspended in albumin (10 mg/100 ml) buffer. The concentration of bacteria was estimated from a constructed curve of transmittance at 600 nm and confirmed later by quantitative plating in agar.

*Phagocytosis and killing of staphylococci.* The phagocytic system was essentially as described previously (13). Briefly, $2.5 \times 10^7$ 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 incubator model 6250 (Eberbach Corp., Ann Arbor MN), 100 reciprocations per min. After ~ 5 min 0.2 ml of an overnight culture of Staph was added (zero time) at Staph/PMN (or of Staph/cytoplast) ratios of 19–28:1, and the shaking incubation continued. After 20 min and again after 60 min of incubation, duplicate samples were sedimented, their supernatants were reserved, and the resuspended cells or fragments were disrupted by Teflon-glass homogenization (1 min, ice-water bath). The remaining live bacteria, both in supernatants and associated with cells or fragments, were enumerated by duplicate plating in agar, and compared to the inoculum. Bacterial controls, incubated without cells or fragments ("serum controls"), were also enumerated. Other samples at 60 min were smeared and stained (Wright's stain), and 200 consecutive cells or fragments were examined for numbers of associated bacteria.

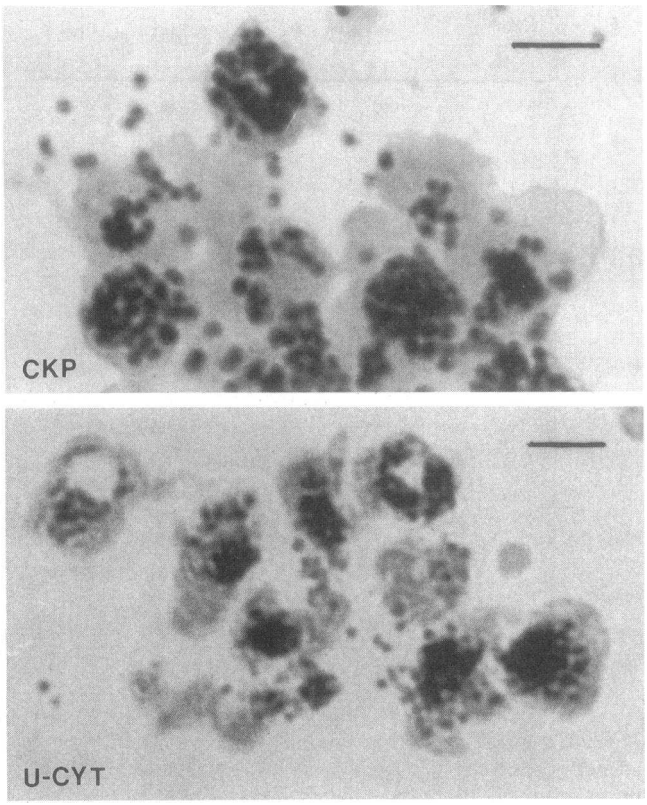


*Figure 3.* Uptake of staphylococci by cytoplasts after cryopreservation. Smears were made of CKP and of U-CYT sampled after 60 min of incubation with bacteria. They closely resemble fresh cytoplasts given bacteria (not shown). Staph/CKP = 36:1. Staph/U-CYT = 49:1. Wright's stain. Bars, 5 $\mu$m.

## Results

*Cryopreservation.* After freezing and thawing, recovery of CKP and of U-CYT is about 70%, of which 98–99% exclude trypan blue dye. In contrast, PMN are largely destroyed. Despite the latter, we refer to this procedure as "cryopreservation" to distinguish it from rapid freezing (in ethanol/acetone) and thawing without dimethyl sulfoxide, which disrupts both PMN and cytoplasts (11, 12).

*Motile function.* Like their fresh counterparts (7, 12), both types of cytoplasts after cryopreservation adhere to glass, spread, and exhibit membrane movement under direct microscopic observation. They preserve their characteristic locomotory appearance (7, 12), the CKP riding higher on the substratum (i.e., being less generally adherent) than the U-CYT (Fig. 1). Both cytoplasts retain their chemotactic responsiveness in a gradient of fMet-Let-Phe (Fig. 1). In order to indicate directed locomotion (chemotaxis) in still photographs taken from videotape, fields were chosen in which most cytoplasts had "tails": temporary aggregations of the most adherent parts of the membrane that form as the cytoplast moves away from them; tails point away from the direction of locomotion.

*Activatable respiratory burst oxidase activity.* Like their fresh counterparts (12), virtually all U-CYT after cryopreservation are able to reduce NBT dye to formazan, i.e., they retain evidence of respiratory burst oxidase activity, although in a variable minority of fragments NBT reduction is punctate rather than diffuse. As a measure of oxidase activity of the fragments as a whole, we also examined the generation of superoxide anion ($O_2^-$) by U-CYT on stimulation by PMA or by fMet-Leu-Phe (Fig. 2). Except for a brief lag period in response to PMA, cryopreserved U-CYT behaved like fresh ones.

Fresh CKP, as a result of the brief heating that stimulates their formation, do not reduce NBT dye or generate $O_2^-$ on stimulation with appropriate agonists (8, 9), and, not surprisingly, neither do CKP after cryopreservation (Fig. 2).

*Phagocytosis and killing of staphylococci.* Like their fresh counterparts (11, 12) both cytoplasts readily take up staphylococci (Fig. 3), and the distribution of bacteria among them (i.e., numbers of staphylococci per cytoplast) is essentially the same before and after cryopreservation (Fig. 4). The histograms of these fragments are somewhat skewed to the left compared to that of their larger, fresh parent PMN (Fig. 4). However, after cryopreservation the uptake and distribution of

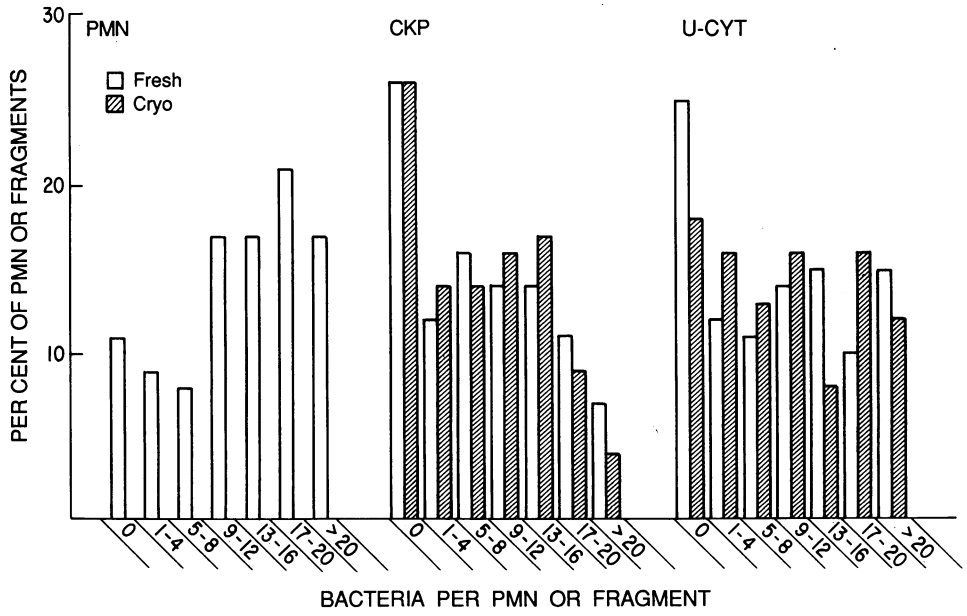


*Figure 4.* Distribution of bacteria among cytoplasts before and after cryopreservation. Aliquots of fragments from the same parent PMN were incubated with live staphylococci for 60 min, and then 200 consecutive cells were examined. Ratios: Staph/PMN or fragment were 23:1 before, and 28:1 after cryopreservation. Distributions before and after were similar for both CKP and U-CYT. The distribution for parent PMN after cryopreservation (not shown) is unreadable because of gross disintegration of the cells.

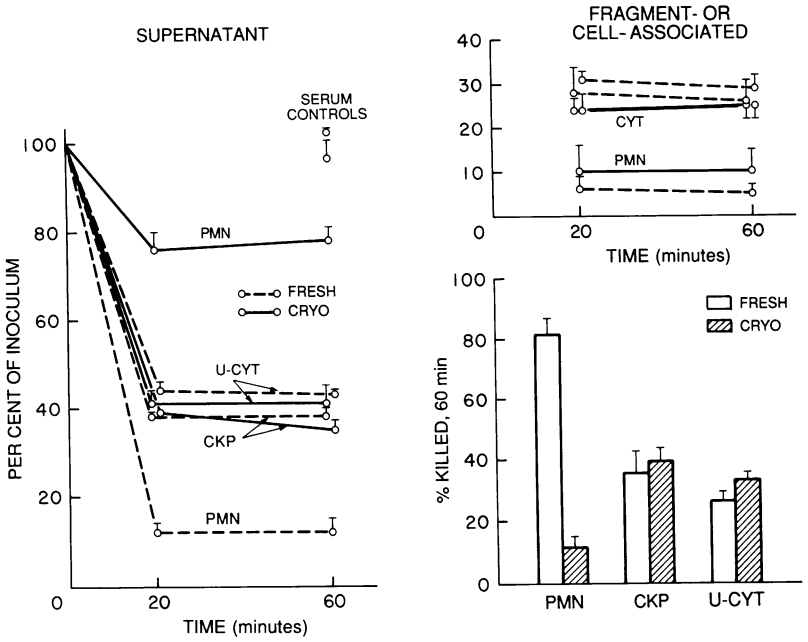


*Figure 5.* Uptake and killing of staphylococci by cytoplasts and by their parent PMN before and after cryopreservation. Results have been combined from three separate experiments in which duplicate samples were taken after 20 minutes of incubation of cytoplasts or parent PMN with staphylococci, and again after 60 min; means + SEM are indicated. Fresh cytoplasts remove bacteria from the medium (*supernatant, left panel*) less efficiently than their larger and granule-rich parent PMN but, unlike the parent cells, are able to maintain essentially all this activity after cryopreservation. Percentages of live, fragment-associated bacteria are also similar before and after cryopreservation (*top, right*), as is percent killing at 60 min (*bottom, right*); the latter is derived from the line drawings: 100- (supernatant + fragments). Ratios: Staph/PMN or fragments for the three experiments were 16:1, 23:1, and 19:1 before, and 27:1, 28:1, and 16:1 after cryopreservation.

bacteria among PMN is unreadable because of gross disintegration of the cells.

The uptake data are confirmed independently in studies of the recovery of live staphylococci, determined by quantitative plating in agar, after incubation with fragments or with their parent PMN (Fig. 5). Fragments are able to remove staphylococci from the medium (supernatant; left panel) as well after cryopreservation as before. (This is in sharp contrast to cytoplasts frozen and thawed rapidly without DMSO (not shown); they resemble serum controls [11, 12]). The larger and granule-rich parent PMN remove bacteria more efficiently than fragments when fresh, but very poorly after cryopreservation, when we know they are largely disrupted. Killing follows uptake (Fig. 5, *lower right*): fragments kill staphylococci as well after cryopreservation as before; the parent PMN, much less well.

## Discussion

Both CKP and U-CYT behave essentially the same after cryopreservation as before. They adhere, spread, locomote, and respond chemotactically to the formyl peptide fMet-Leu-Phe (Fig. 1). The cytoplast with activatable oxidase activity (U-CYT) maintains that capacity among individual fragments (by NBT reduction) and in the aggregate (by generation of superoxide anion, Fig. 2). Both cytoplasts take up staphylococci with undiminished avidity and similar distribution before and after cryopreservation (Figs. 3–5), and they kill ingested bacteria equally well (Fig. 5). This preservation of form and function compared to the parent PMN is almost surely due to their paucity of granules (2–5).

A more difficult question is how these cytoplasts, fresh or preserved, kill bacteria as well as they do, and especially the CKP, which lacks both granules and activatable oxidase activity, both of which are thought necessary for optimal killing of catalase-positive organisms such as staphylococci (19). Three possibilities might be considered, none of which are entirely satisfying. First, rare granules may be sufficient to provide killing by nonoxidative (at least for CKP) mechanisms (20), without affecting cryopreservability. In view of the distribution of bacteria among cytoplasts and the magnitude of killing, we tend to doubt it. Secondly, known killing components may be acting in different places: e.g., granule contents translocated to plasmalemma during production of fragments, as has been indicated in other cytoplasts (21). Again, this "killer membrane" would have to be extremely efficient. Finally, other unknown killing components, as perhaps might be induced by heat in the case of CKP, might be operating. Evidence on this score is currently lacking, although PMN can produce heat-shock proteins (22).

Whatever the mechanism(s) of killing, this cryopreservable capacity, along with the ability to seek (through chemotaxis) and ingest bacteria, raises the possibility of using stored fragments in patients whose own PMN are dysfunctional or lacking due to disease or chemotherapy. In some situations one can envisage autologous fragments being collected and stored for periods of crisis, providing bacterial killing power without immune complications. Of course the current work is only a first step; there are, for example, problems of scale—yields need to be improved—as well as the necessity of showing that fragments will work in vivo as well as in vitro. Nonetheless, here is preliminary evidence that a cryopreservable "neutrophil surrogate" may be in the wings.

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