

# Structures Suggesting Cell-Wall-Deficient Forms Detected in Circulating Erythrocytes by Fluorochrome Staining

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Cell-wall-deficient (CWD) forms of bacteria are associated with certain cases of idiopathic septicemia. In this preliminary study of blood examined immediately after venipuncture, structures with a morphology characteristic of CWD forms were seen parasitizing the erythrocytes. These inclusions were usually circumferential, but in some cases they protruded from the red cells. The CWD forms were detected by staining with Gould's rhodamine-labeled muramidase, which reacted similarly to acridine orange but with greater specificity. A blocking test, employing unlabeled muramidase, indicated the specificity of the reaction between muramidase and the microbial substrate. Reaction of the forms with muramidase indicates their bacterial, rather than mycoplasmal, nature. Thus *in vivo* CWD forms have a detectable component of muramic acid, at least in certain cases. Sixty-eight individuals with a diagnosis of fever of unknown origin were tested, with 51 nondebilitated individuals serving as controls. More intraerythrocytic forms reacting with muramidase were found in the patients than in the controls. Nearly 40% of the cases had a relatively high incidence of erythrocyte parasitism. In some instances when freshly drawn blood was examined, the structures, which appear to be microbial, extended in rhizoid filaments from the erythrocytes.

Studies by others (19) and by ourselves have shown that classical bacteria found in septicemia are occasionally seen in smears of peripheral blood. Initially, this study was undertaken to ascertain whether leukocytes in smears of peripheral blood of febrile patients might reveal cell-wall-deficient (CWD) forms, thus giving a diagnostic clue to the septicemic state. Preliminary studies, however, indicated that recognition of small intraleukocytic CWD forms was impossible because of the presence of intracellular structures characteristic of the white blood cell when stained by either Wright's stain or acridine orange.

However, intraerythrocytic parasitism was striking and, therefore, chosen for further investigation. Previous studies by Seibert et al. (18), Mule (9), Wuerthele-Caspe et al. (22), Tedeschi et al. (20), Dmochowski et al. (3), and Villequez (21) indicated that microbial forms may often be found within erythrocytes during both the infectious and the nondebilitated state of man and other vertebrates. However, most studies documented evidence of such

structures only after various incubation periods in liquid media.

A high number of all blood cultures submitted to bacteriology laboratories are negative. However, more positives are found when CWD bacteria are sought (1, 4, 7, 8, 12); sometimes the increase is at least threefold (10). Therefore, early detection of the CWD variants is of practical importance, since the seriousness of septicemia necessitates a prompt clue as to the infectious state.

The purpose of this research was to employ a simple laboratory procedure for the rapid detection of CWD forms in freshly drawn blood. Goals were twofold: first, to obtain a specific stain for the microbial cell wall substrate; and, second, to apply this stain for the recognition of both intra- and extraerythrocytic microbial growth.

## MATERIALS AND METHODS

**Selection and processing of blood specimens.** Blood samples from febrile patients suspected of septicemic involvement, all drawn in tubes con-

taining liquoid (sodium polyanethyl sulfonate), were received from four metropolitan hospitals. Approximately 8.0 ml of the patient's blood, withdrawn by venipuncture, was placed in 1.7 ml of sterile liquoid. All bloods were refrigerated within 30 min and examined within 1 hr. Tests for classical bacteria were made both in the hospital and in our research laboratory. Samples which yielded classical bacteria during the 2 weeks of incubation were eliminated from the study. Sixty-eight blood samples were negative for classical bacteria and comprised the test bloods of the series.

The controls were from physically healthy individuals who had requested a physical examination. They varied in age from those in the premarital group to individuals in retirement homes. The specimens were drawn in tubes containing sterile liquoid at two clinical laboratories. The bloods were examined by the same procedures employed for hospitalized patients.

**Fluorescence staining and microscopy.** To reveal nucleic acid-containing structures, acridine orange was applied in the following manner: 0.003 ml of the anticoagulated fresh blood was suspended in an equal volume of a working solution of acridine orange stain on a Gold Seal slide (Clay-Adams, Inc.), covered with a no. 1 cover slip and examined with a Zeiss fluorescence microscope, employing a 32-mm BG 12 exciter filter and OG 4 barrier filters.

A 1-ml amount of the blood was aseptically diluted in 1.0 ml of sterile Tryptose phosphate broth (Difco), and 0.003 ml of this broth-blood mixture was added to an equal volume of rhodamine B-labeled muramidase on a Gold Seal slide. A cover slip was applied, and, after incubation at room temperature for 5 min, the preparation was examined with the fluorescence microscope, as was the acridine orange preparation. Dilution of the erythrocytes in the Tryptose phosphate broth resulted in an even suspension of cells and eliminated clumping.

To substantiate the specificity of the labeled muramidase, a duplicate series of specimens was examined, employing pretreatment with unlabeled muramidase before the labeled enzyme was applied.

A lysozyme-sensitive strain of *Bacillus megaterium* was used as the positive control for the activity of the labeled muramidase. For this purpose, an 18-hr culture of the bacillus was suspended in a sterile 6% sucrose solution. A 0.003-ml fraction of this suspension was added to an equal volume of labeled muramidase and allowed to stand at room temperature for 15 min before examination with the fluorescence microscope.

The basic methods used in preparation of the acridine orange and labeled muramidase are described, respectively, in reports by Chattman et al. (2) and Gould et al. (5).

To eliminate blood cultures containing classical growth and to follow the morphological growth patterns of CWD forms in culture, 0.1-ml samples of the fresh blood were inoculated, in duplicate, into 5.0 ml of Tryptose phosphate broth containing 0.05% agar. All cultures were incubated in a water-bath shaker at 37 C for 14 days.

At 2, 7, and 14 days, 0.1-ml samples were drawn from each culture. Samples (0.003 ml) of the culture were suspended in an equal volume of the working acridine orange and also in an equal volume of the rhodamine B-labeled muramidase. These slides were then examined with the ultraviolet microscope. To check for classical bacterial growth, the remaining culture samples were inoculated on blood-agar plates, which were incubated with 2% CO<sub>2</sub> aerobically and anaerobically.

**RESULTS**

Freshly drawn blood from the hospital patients demonstrated a relatively high incidence of parasitized red cells compared to parasitism found in the normal controls (Table 1). Thus, 43% of the normal people had no demonstrable intraerythrocytic CWD forms, whereas only 10% of the patients failed to demonstrate this phenomenon. Accordingly, failure to find parasitized erythrocytes on such direct examination would weigh against active bacteremia. Or, alternately, two or more parasitized cells per microscopic field could be considered highly suggestive of bacteremia, since this incidence occurred in 39.7% of the patients and only 3.8% of the controls.

In each examination, the readings of the microbial morphological patterns developed with acridine orange agreed with those for the muramidase reaction. However, brighter patterns were obtained with the labeled muramidase. Parasitism consisted of from one to seven structures, approximately 1.1 μm in diameter, mostly attached to the inner periphery of the erythrocyte (Fig. 1a and 1b).

In freshly drawn blood, each field of approximately 300 erythrocytes contained from zero to

TABLE 1. Initial examination of freshly drawn blood

Erythrocytes parasitized intracellularly (no./field)	Hospital patients <sup>a</sup>	Nondebilitated individuals <sup>b</sup>
None observed . . . . .	7 (10.3)	22 (43.1)
Less than one/field . . . . .	15 (22.1)	15 (29.4)
One/field . . . . .	19 (27.9)	12 (23.5)
Two/field . . . . .	10 (14.7)	1 (1.9)
Three/field . . . . .	5 (7.4)	1 (1.9)
Four/field . . . . .	4 (5.9)	0
Five/field . . . . .	3 (4.4)	0
Six/field . . . . .	4 (5.9)	0
Seven/field . . . . .	1 (1.4)	0

<sup>a</sup> Values in parentheses are expressed as percentages. Incidence of two or more parasitized cells per microscopic field = 39.7%.

<sup>b</sup> Values in parentheses are expressed as percentages. Incidence of two or more parasitized cells per microscopic field = 3.8%.

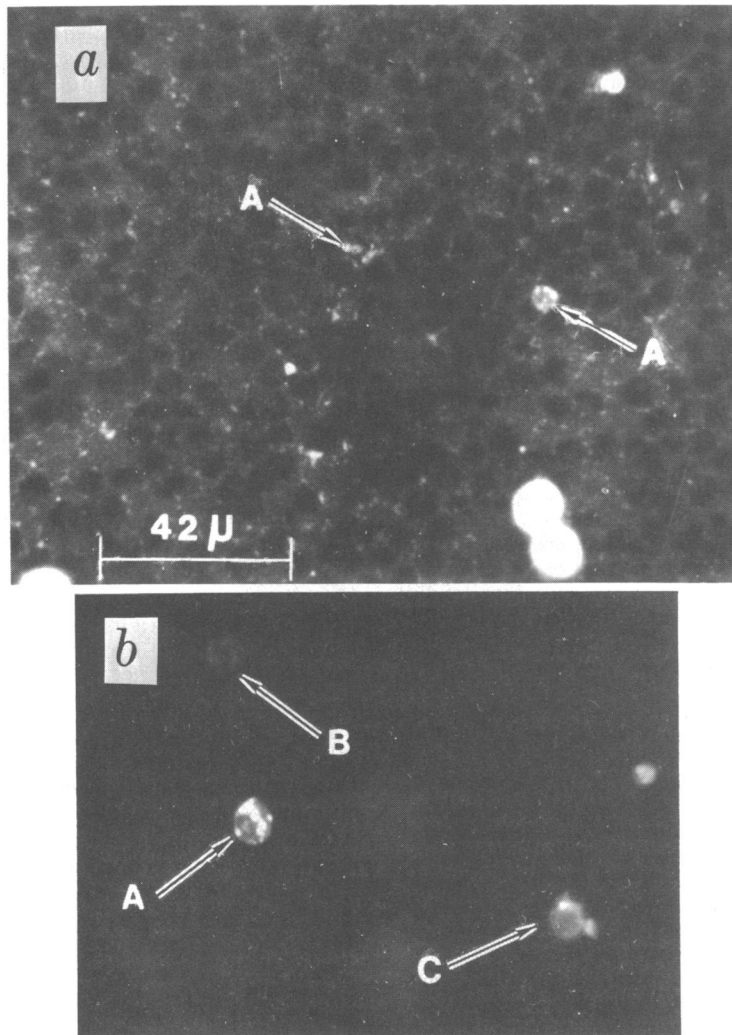


FIG. 1. (a) Fresh blood initially examined with acridine orange. Two red blood cells (A) containing CWD forms which stained orange. Black spheres are red blood cells. Large spheres are white blood cells which stained orange. Magnification, 500X. (b) Fresh blood initially examined with labeled muramidase. Red blood cells (A and B) containing CWD forms which were stained yellowish-white. Protrusions of CWD forms (C) from a red blood cell.

six parasitized erythrocytes; rarely were seven parasitized cells per field observed. On this initial examination, the blood from five hospital patients showed extensive growth protruding from erythrocytes.

When examined after 14 days of incubation, the blood of approximately 82% of the patients and 43% of the controls exhibited increased microbial growth. This growth included an increase in intracellular forms, extension of intracellular structures to protrude through the cell membrane and extend extracellularly, extracellular budding spheres, sphere clusters,

and single spheres. A few rodlike structures were also present (Figs. 2-5).

Continued subcultures were made in duplicate: one with agitated and one with stationary incubation. In these tubes, obvious macroscopic growth was ultimately obtained by either method of incubation. In some instances, as many as six successive subcultures were made. When small inocula were used in serum-enriched pour plates, numerous colonies developed in most instances. The growth obtained was typical in morphology and in staining reactions to CWD forms which consti-

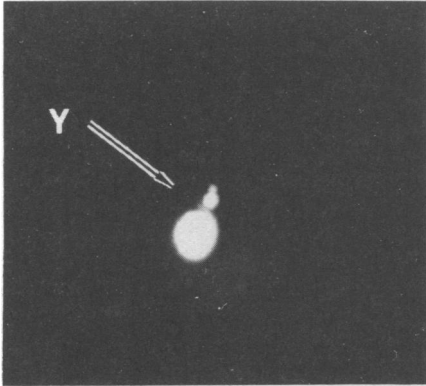


FIG. 2. Fourteen-day blood culture examined with labeled muramidase. A budding spheroplast (Y).

tute the well known granular colonies of *Proteus mirabilis*, which can be formed in penicillin-containing media totally lacking serum. Controls for sterility of the media were always included, and the tubes and plates were checked for structures before incubation to assure that the forms found after incubation represented new development.

The numerous subcultures which may result in reversion to classical bacteria were not done in this series since duplicate samplings of blood were not accessible from most patients. Before any revertant can be considered of proven origin from a patient, we believe that it must be found in sequential blood cultures from an individual.

### DISCUSSION

Preliminary studies involving examination of 200 samples of blood were conducted to become acquainted with the artifact content of erythrocytes of nondebilitated individuals (6, 10, 15, 16). For these studies, acridine orange was used to detect deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). The size and morphology of intracellular erythrocyte debris, such as Howell-Jolly bodies, reticulocytes, Cabot rings, Pappenheimer bodies, ribosomal fragments, and Heinz bodies (17), were significantly different from those found with CWD forms, when stained with acridine orange.

Since many immature erythrocytes contain DNA, the possibility existed that residual DNA may also be present in the mature erythrocyte. Thus, the fluorescent muramidase was employed to expedite a clear delineation between erythrocyte nuclear material and CWD forms. Gould used this rhodamine B-muramidase complex to identify the cell wall sub-

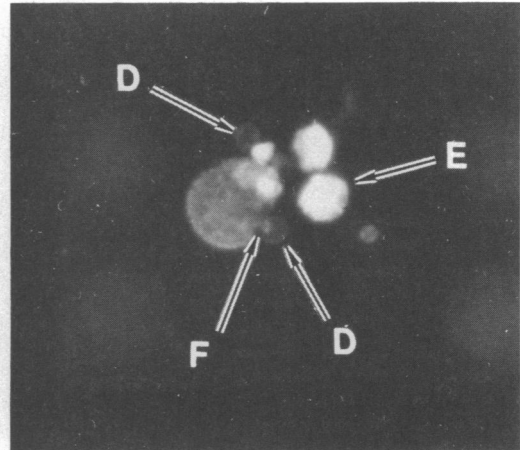


FIG. 3. Fourteen-day blood culture examined with acridine orange. Two red blood cells (D) from which a series of large interconnecting spheroplasts (E) have grown. Note the intimate cellular bridges (F) connecting the several structures.

strate in mature bacterial spores, where it successfully penetrated the dense spore coat. It was therefore postulated that the muramidase might penetrate the erythrocyte membrane and become affixed to the underlying microbial substrate, if present.

However, the possibility existed that strongly hydrophobic organic groups attracted the rhodamine molecule, along with its attached enzyme, to a site other than the specific muramic acid substrate. To confirm the specificity, the specimens were pretreated with unlabeled muramidase. In all such control studies, aberrant microbial structures were not observed with the labeled muramidase stain. This blocking of the reaction indicated the specificity of the labeled enzyme for its cell wall substrate.

Highly significant quantitative differences were found between erythrocyte parasitism in patients and in nondebilitated individuals. In febrile patients, the two or more parasitized red cells per field, so frequently seen, indicated a significant increase of aberrant microbial forms over those found in nondebilitated individuals. This increase suggested septicemia even though no classical bacteria were present. Since these forms stain with labeled muramidase, they appear to be CWD forms of bacteria rather than mycoplasma.

Table 1 indicates that approximately 57% of healthy individuals have circulating erythrocytes which host CWD forms. Possible variation which may occur with age, occupation, etc., could make an interesting study. In this

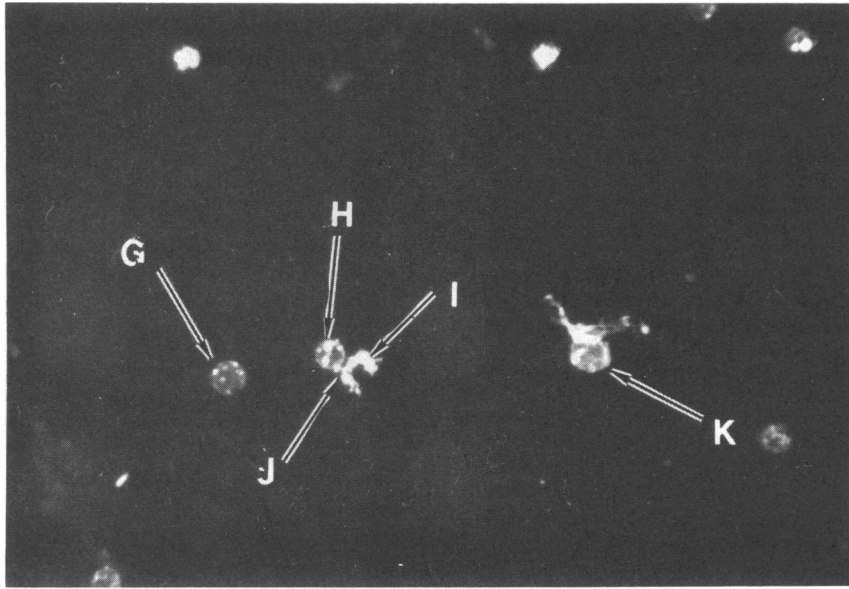


FIG. 4. Fourteen-day blood cultures examined with labeled muramidase. A red blood cell (G) with intracellular growth of CWD Forms. Intracellular growth of CWD forms (H) with an extracellular colony (I) attached to the red blood cell by a slender bridge (J). Another internally and externally parasitized red blood cell (K).

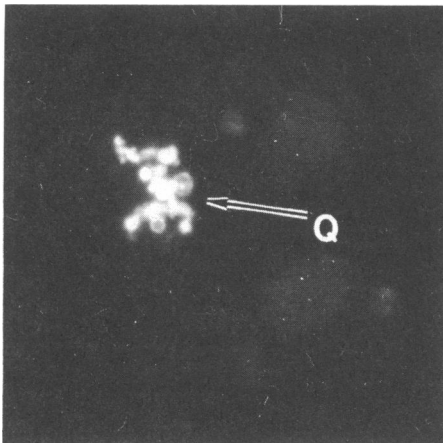


FIG. 5. Fourteen-day blood culture examined with acridine orange. A staphylococcal-like cluster of spheres (Q).

small series, it was evident that at least some of the infected normals were young people in apparent excellent health. Documented evidence by Pease (13, 14), Tedeschi et al. (20), and Villequez (21) indicates the presence of CWD forms in the blood of healthy individuals. In repeated studies of sections of microorganisms examined with the electron micro-

scope, the forms reported by Tedeschi have never demonstrated the presence of a cell wall. Thus, it appears that, in addition to muramidase-staining forms in 57% of the population, there is an omnipresent organism lacking a wall, which he currently regards as a mycoplasma or stable L-form.

The implication of the presence of CWD forms in the blood of normal people is of great interest. Additionally significant is the question, if CWD forms are normal flora of the blood, do the same organisms ever increase in number to cause acute infections or is septicemia always a function of a new invasive pathogen?

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