Phagocytosis of Microbial L-Forms

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Received for publication 4 April 1972

Little data are available regarding L-form phagocyte interactions. Due to difficulty with more conventional methods, a serum-free technique of surface phagocytosis employing cellulose ester micropore filters, fluorochrome staining, and reflecting fluorescence microscopy was developed. Intracellular localization of L-forms was confirmed by electron microscopy. In timed experiments, phagocytosis of a group D Streptococcus (ATCC 23241) and its L-form (T531) were maximal at 30 min, although the bacteria were consistently better phagocytized. Phagocytosis of an Escherichia coli (strain Yale) and its L-form was also demonstrated with the same technique. The lesser phagocytosis of L-forms may be related to their lower chemotactic activity.

Phagocytosis by polymorphonuclear (PMN) leukocytes is an important defense against an infecting microorganism. Several investigators have postulated a pathogenic role for microbial L-forms (3, 7, 8, 10), but only a few studies of L-form-phagocyte interactions have been published. These have been difficult to interpret, owing either to reversion of the organism to the bacterial form during the experiment (13) or to insufficient data (P. Charache and L. W. Marshall, Abstr., Clin. Res. 18:437, 1970).

Our previous attempts to demonstrate phagocytosis of L-forms have been hampered by the need of many of these cell wall-deficient organisms for hypertonic surroundings, an environment which adversely affects PMN leukocyte phagocytic activity (4). Further, the usual in vitro reaction of PMN leukocytes and microorganisms employs serum or other opsonins, which may be bactericidal for L-forms (11) or have an adverse effect upon their structural integrity. These factors make conventional viable count methods interpretable and add to the difficulty of identifying L-forms in ordinary Giemsa-stained smears.

In preliminary experiments employing a simple tube reaction, L-form engulfment could not be seen either on stained smears or during periods of prolonged observation by interference microscopy of hanging drop preparations. To enhance the phagocytic activity of the leukocytes, we employed the technique of surface phagocytosis (18). As poor L-form adherence to the usual surfaces was seen, a cellulose ester micropore filter was used. The thinness of the filter and its relatively uniform surface allowed examination of the reaction mixture both by fluorochrome staining with reflected fluorescence microscopy and by ultrathin section electron microscopy. Phagocytosis of a group D Streptococcus and its L-form was studied in detail. Studies were also performed with a strain of Escherichia coli and its L-form.

MATERIALS AND METHODS

The organisms employed were Streptococcus faecalis strain GK (ATCC 23241), its nonreverting L-form (T531), E. coli strain Yale, and its nonreverting L-form. These organisms were chosen because all four can be grown in broth without added osmotically active substance. The parent bacteria were both isolated from the urine of patients with pyelonephritis. Media employed throughout were Brain Heart Infusion broth and agar (Difco). Both PMN leukocytes and microorganisms were washed and then suspended in Hanks balanced salt solution (Microbiological Associates, Inc.) with 0.02% bovine serum albumin (Hyland Laboratories) and 50 mg% glucose (HBG).

Leukocytes were obtained from 12 ml of normal human peripheral blood by sedimentation for 1 hr with 4 ml of 6% dextran (molecular weight 204,000; Sigma Chemical Co.) in 0.9% saline and 0.5 ml of 1% heparin (Calbiochem). The buffy coat was decanted and centrifuged at 130 × g for 5 min; the cells were then resuspended in HBG to a concentration of 10⁷ cells per ml. These were kept in ice water until ready for use (within 1 hr). No serum was added to the mixture.

 Cultures in early stationary growth phase were prepared from the parent bacteria (18 hr) and the L-forms (24 hr). Bacteria and L-form cultures were iced and centrifuged at 5,000 × g for 20 min at the same time. After resuspension in HBG at appropriate dilutions, reaction mixtures consisting of 10⁷ PMN leukocytes per ml and 10⁸ microbial cells per ml were prepared.
PHAGOCYTOSIS OF MICROBIAL L-FORMS

These mixtures, along with leukocyte and organism controls, were spread in volumes of 0.1 ml over the surface of prewarmed 25-mm cellulose filters (0.45 μm pore size; Millipore Corp.) on gauze pads soaked in HBG. Incubation was carried out in a humidified atmosphere of 5% CO₂ and 95% O₂ at 37 C.

Filters were removed from the incubator after periods from 15 to 90 min and fixed by immersion in iced 0.5% glutaraldehyde. A portion of each filter was used for fluorescence microscopy and another portion was prepared for electron microscopy.

The portion of the filter for fluorescence microscopy was stained with acridine orange (Allied Chemical Co.), diluted 1:10,000 in pH 6.4 deionized water for 30 min at room temperature, and mounted with a cover slip on a glass slide.

The portion of the filter for electron microscope examination was further fixed with osmium tetroxide and stained with uranyl acetate and lead. Portions of the filter were dehydrated in ethanol, treated with styrene, and embedded in Vestopal (Jaeger, Geneva). Ultrathin sections were made with a diamond knife.

Phagocytosis on the filters was revealed by fluorescence microscopy and evaluated quantitatively by examination of 200 PMN leukocytes; the number of leukocytes which had phagocyted and the number of microorganisms which had been engulfed were recorded.

RESULTS

Examination of the filters with reflected ultraviolet light from a mercury vapor lamp through Zeiss BG38 and BG12 exciter filters with a Zeiss 47 barrier filter showed the following: nuclei of leukocytes fluoresced bright orange, their cytoplasm a bright green; the bacteria and L-forms fluoresced a brilliant uniform orange. Figures 1a, 1b, and 1c are photomicrographs of leukocytes alone, leukocytes reacted with T531, and leukocytes reacted with the parent bacteria. These demonstrate the intracytoplasmic location of the microorganism. Figure 2a is a representative electron micrograph of an L-form and PMN leukocyte reaction mixture. In this section, a large L-form is seen within the cytoplasmic membrane of a PMN leukocyte. Its morphology is more regular than that of the L-forms seen outside the cytoplasmic membrane. It appears to be somewhat swollen and is surrounded by a three-layered membrane closely applied to its intrinsic three-layered unit membrane.

Results from a typical timed experiment are shown in Table 1. Both the L-forms and the parent bacteria follow the same general pattern, with significant phagocytosis first recorded at 15 min, reaching a maximum at 30 min, and declining variably thereafter. A marked deteriora-

PMN leukocytes in both reaction mixtures in which microorganisms were present, suggesting these PMN leukocytes have been "activated."
Fig. 2a. Portion of cytoplasm of PMN demonstrating an intracellular L-form (Li). Lysosomes (arrows) are seen surrounding the organism. Note the characteristic pleomorphic appearance of the extracellular L-forms (Le). The relatively large size of the intracellular L-form could be due to division or to swelling preceding lysis. × 21,000.
tion of PMN leukocyte cellular morphology was seen in association with this decline during both L-form and bacterial experiments, PMN leukocytes incubated alone for the same time period showed no significant changes in morphology, suggesting that some cytopathic effect was associated with the presence of the microorganism. At 30 min (the time of maximal phago-

FIG. 2b. Enlargement from 2a illustrating the intact membranes of the L-form (ML) and the phagocytic vacuole (MV). The texture of the material in the triangular space between these membranes is characteristic of the PMN cytoplasm. X67,000.
TABLE 1. Phagocytosis of Streptococcus faecalis GK and L-form (T53I) at various time periods

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>S. faecalis L-form (T53I)</th>
<th>S. faecalis GK</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per cent PMN leukocytes phagocyted</td>
<td>No. of microorganisms per phagocyte</td>
</tr>
<tr>
<td>15</td>
<td>23</td>
<td>1.5</td>
</tr>
<tr>
<td>30</td>
<td>53</td>
<td>3.6</td>
</tr>
<tr>
<td>45</td>
<td>41.5</td>
<td>2.0</td>
</tr>
<tr>
<td>60</td>
<td>35</td>
<td>2.7</td>
</tr>
<tr>
<td>90</td>
<td>43</td>
<td>2.8</td>
</tr>
</tbody>
</table>

a Ratio of polymorphonuclear (PMN) leukocytes to T53I, 1:12.4.
b Ratio of PMN leukocytes to S. faecalis, 1:11.8.
c Two hundred cells counted by reflected fluorescence microscopy.

cytosis), results of triplicate experiments with PMN leukocytes from one donor varied for T53I from a low of 44% PMN leukocytes phagocytizing with 1.8 organisms per phagocytizing cell to a high of 53%, with 3.6 organisms per phagocytizing cell. Simultaneous experiments with the parent bacteria varied from 54.5% PMN leukocytes phagocytizing with 2.67 organisms per phagocytizing cell to 79% with 5.6 organisms per cell. Similar observations were made regarding phagocytosis of E. coli (strain Yale) and its penicillin-derived nonreverting L-form. Five other PMN donors were used for repeat experiments with the S. faecalis L-form and parent. Results varied from 35 to 83% leukocytes phagocytizing for the L-form and from 50 to 98.5% phagocytizing for the parent bacteria.

Experiments were also performed to evaluate the effect of growth phase of the microorganism upon its phagocytability. Within the range of variation noted above, no consistent differences could be seen between organisms in early log phase and early stationary phase. All the L-forms and bacteria employed in these tests survived in HBG for over 2 hr. Owing to the possibility that the L-forms are susceptible to lysis by the Triton X-100 detergent found in the usual cellulose ester filters, experiments were repeated with Triton-free filters (Millipore Corp.); results were comparable. Attempts to remove quantitatively the phagocyte-microorganism mixture from the filters to determine viability of the intracellular L-forms have not been successful.

In parallel experiments performed at the same time and with the same ratio, bacterial forms were always more readily engulfed than were their derived L-forms. A possible explanation for this difference arose from preliminary experiments comparing the chemotactic activity of the L-forms to that of the bacteria. Using the same medium, cell donors, and PMN leukocyte to microorganism ratio (1:10), PMN migration in Boyden chambers (2) after 3 hr was considerably less when the L-forms were used as attractants than when bacterial forms were used.

DISCUSSION

The phenomenon of surface phagocytosis was described both in vivo and in vitro by Wood et al. in 1946 (18). The surfaces employed were both living and fixed rodent lung, fibrin clots, filter paper, and more recently glass slides (5). In 1965, Vaughan (17) described the use of cellulose ester micropore filters. After fixation and conventional staining, the filters were cleared with xylene and examined by transmitted light microscopy. The choice of acridine orange staining and reflected fluorescence microscopy in the present study was based upon the ease with which the L-forms and bacteria could be distinguished from each other and from elements with the PMN cytoplasm. The staining reactions of the PMN leukocytes to acridine orange which we observed are at variance with those described by Armstrong (1); Chattman et al. used acridine orange to identify L-forms, but also described different color reactions (3). These differences are probably related to the pH and concentration-dependent nature of the staining reaction (1, 16), the fixative employed, and the varying composition of the dye as produced by different manufacturers. (The controversy over the nature of the staining reactions of acridine orange and orange red is discussed at length by A. G. E. Pearse in his book, Histochernistry, Theoretical and Applied, 2nd ed., 1960, London, Churchill, p. 731-733. As shown in Fig. 1b, the fluorescing L-forms, though pleomorphic, were relatively large and easily resolved by a 100×, numerical aperture 1.40 objective. Depth of focus with this lens is less than 0.3 μm (19), suggesting strongly that organisms revealed within the boundaries of the PMN cytoplasm are indeed intracellular.

As the L-forms are bounded by a single triple-layered membrane (14), the electron micrograph showing the six-layered membrane surrounding the L-form suggests the presence of the microorganism within an intracellular vacuole.

Unfortunately, our present inability to remove the reaction mixture from the filter has prevented any simple microbiological assessment of the viability of the phagocytosed L-forms.

Others have studied the relationship of L-forms to nonphagocytic mammalian cells. L-forms of
group A streptococci (15) and Brucella (8) have been shown to assume an intracellular position when introduced into human embryonic fibroblast and hamster kidney tissue cultures, respectively. Cytopathic effects from these and other L-forms have been reported by several investigators using other cell culture lines (10, 12, 15). Whether these effects are secondary to depletion of an essential metabolite, as is the case with arginine-splitting mycoplasmas (9), or due to more direct cytotoxic mechanisms, is not known, but in the case of Brucella, the L-forms are significantly more toxic to the cell culture line than to the parent bacteria (6). Whether bacteria without cell walls may be responsible for disease is as yet unknown, but L-forms of Proteus have been shown to persist in the urine of patients with chronic urinary tract infections after treatment (7). A "protected" hypertonic environment such as the renal medulla or the interior of a mammalian cell would seem to be an ideal place in which an otherwise fragile form might survive.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grants AI 2557 and AI 3310 from the National Institute of Allergy and Infectious Diseases and HE 1114 from the National Heart Institute.

LITERATURE CITED