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Modulation of Macrophage Lysosomal pH by *Mycobacterium tuberculosis*-Derived Proteins

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Macrophage lysosomal pH was significantly (>1 pH unit) increased in a reversible, concentration-dependent manner characterized by a saturable and cyclic kinetics after exposure to culture filtrate protein extract derived from *Mycobacterium tuberculosis*. Lysosomal alkalinization peaked 30 min after administration of culture filtrate protein extract to cells of the macrophagelike cell line J774A.1. The alkalinization was reversible, and a second peak was observed approximately 60 min after incubation. Maximum lysosomal alkalinization increased as a function of culture filtrate protein extract concentration, reaching an apparent saturation level around 700 to 1,000 μg/ml, although the time course for this process was not significantly dependent on antigen concentration. The alkalinizing agent(s) was heat labile and produced a similar effect in cells which had a different lysosomal enzyme composition. Our observations are consistent with the presence of one or more mycobacterial antigens which have a pH-dependent affinity for lysosomal structures essential for lysosomal acidification and which are able to inhibit this lysosomal acidification.

Certain intracellular parasites are known to alter pH values within specific cellular compartments such as phagosomes (3, 8, 16). The inhibition of phagosome-lysosome fusion by *Mycobacterium tuberculosis* (1) strongly suggests that it may also be capable of blocking the acidification of these compartments. The consequences of such an alkalinization mechanism for bacterial survival within the host cell would include a decrease in the hydrolytic activity of lysosomal enzymes and a probable impairment of the fusogenic activity of either or both of these organelles (lysosomes and phagosomes) (9). Mycobacterial products such as polyanions (5, 7), cyclic nucleotides (18), and ammonia (6) have been reported as modulators of normal phagosome-lysosome fusion, yet the biochemical mechanisms by which these molecules might exert this effect remain unclear.

In an attempt to explore the possible existence of a mycobacterial modulation of macrophage lysosomal pH, a pH-dependent fluorescent probe, fluorescein isothiocyanate-dextran, was used to monitor the lysosomal pH of live cells from the macrophagelike cell line J774A.1 after exposure to a culture filtrate protein extract (CFPE). Our results indicate that CFPE causes a reversible alkalinization of the lysosomal lumen of exposed cells. The alkalinization shows a cyclic behavior, suggesting a threshold value for the minimum pH at which these mycobacterial effectors can be biochemically active.

MATERIALS AND METHODS

Bacterial cultures. *M. tuberculosis* H37Rv (TMC102) was obtained from the School of Biological Sciences, National Polytechnic Institute, Mexico City. Lyophilized bacilli were suspended in protein-free Proskawer-Beck-Youmans medium and subcultured in Lowenstein-Jensen medium to produce seed cultures for the inoculation of 100-ml volumes of Proskawer-Beck-Youmans medium in 250-ml Erlenmeyer flasks. Cultures were maintained at 37°C until a stationary growth phase was reached, that is, until the surface of the Proskauer-Beck-Youmans medium was uniformly covered (between 4 and 8 weeks), at which time bacteria were harvested (14).

Preparation of CFPE. CFPE was obtained as previously described (14). Briefly, culture media were separated from bacterial masses by filtration. Proteins in the filtrate were isolated by precipitation with ammonium sulfate suspended in phosphate-buffered saline (PBS), and dialyzed extensively against PBS. Protein concentration was determined by the method of Lowry et al. (11).

Culture media and reagents. Both mouse monocyte-macrophage J774A.1 and P388D.1 cell lines were grown in Dulbecco modified Eagle medium supplemented with penicillin G (100 U/ml), streptomycin (100 μg/ml), 10% fetal bovine serum, 20 mM HEPES (*N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) and sodium bicarbonate (2.0 mg/ml). Dulbecco modified Eagle medium and fetal bovine serum were purchased from GIBCO laboratories (Grand Island, N.Y.). The medium used during recordings was modified such that its pH (7.2) was maintained at atmospheric CO_2 pressure as follows: Dulbecco modified Eagle medium supplemented with sodium pyruvate (0.55 mg/ml), 10 mM HEPES, and antibiotics as described above. Antibiotics, fluoresceinated dextran with an average molecular weight of 70,000 (FD-70), monensin, bovine serum albumin, and transferrin were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Cell culture conditions. Cells were cultured in 16-mm-diameter standard tissue culture wells (Costar, Cambridge, Mass.) with 2×10^6 cells per well and incubated at 37°C in a humidified 5% CO_2 atmosphere for at least 24 h before the experimental protocol was initiated. Cells were then incubated with medium containing FD-70 at a concentration of 2 mg/ml for 24 h. The cells were subsequently washed two

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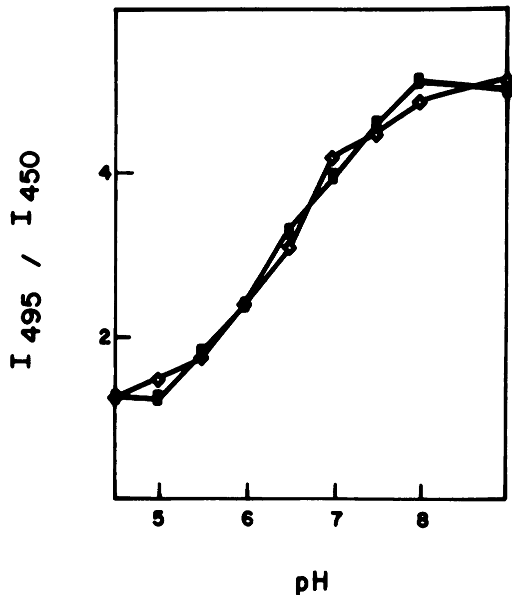

FIG. 1. Calibration curve. Measurements on standard cells (■) and on cells preincubated with CFPE (500 μg/ml) for 30 min (◇).

times with 0.01 M PBS and supplied with fresh FD-70-free medium. All further experimental manipulations were initiated 3 h afterward. Cell viability was determined by trypan blue exclusion (>95%).

Spectrofluorometer recordings. Fluorescence intensity recordings were obtained with an SLM spectrofluorometer. FD-70-loaded cells were incubated in modified medium (see above). At the end of the corresponding incubation period (specific CFPE incubation conditions are stated below for each experimental situation), cells were washed three times with PBS and then supplied with 0.02% EDTA solution. A rubber policeman was used to put cells in suspension; the suspended cells from a single well were placed in a polished glass cuvette (3 by 3 by 12 mm). Each sample was subjected to a 495-nm immediately followed by a 450-nm excitation wavelength beam. Both emission intensities were recorded at a 550-nm wavelength (13). Stimulation times were <5 s in duration, such that photobleaching was minimal.

Calibration curve. FD-70-loaded cells were suspended in Tris-maleate buffered solutions containing 20 μM monensin with a 4.5 to 9.0 pH range. Cells exposed to medium with 500 μg of CFPE per ml 30 min before suspension were also included. Emission intensities at 550 nm were recorded by using excitation wavelengths of 495 and 450 nm as described above (Fig. 1).

CFPE incubation conditions. FD-70-loaded cells were incubated for 5 min at 4°C with modified medium with CFPE (500 μg/ml). Cells were then washed three times with PBS and incubated with fresh CFPE-free medium at 37°C. Measurements were carried out on replicate macrophage cell cultures during the following 90 min. Results are shown as pH values, obtained from referring to the previously recorded calibration curve. Another group of FD-70-loaded cells were incubated with CFPE-modified medium (500 μg/ml) at 37°C for 90 min. Intensity recordings were made during this incubation. A third group of cells was incubated as in the first case (4°C, 5 min), with CFPE heated for 30 min at 56°C. Incubation conditions and measurements for cells from the P388D.1 cell line were identical to those described above. In all cases cell viability was determined by trypan blue exclusion (>90%).

Other protein incubation conditions. Replicate FD-70-loaded J774A.1 cultures were incubated with either transferrin, bovine serum albumin, or CFPE at a concentration of 500 μg/ml. Incubations were carried out as described above (4°C, 5 min).

Analysis of results. Results are expressed as changes in pH relative to the baseline value: pH = (experimental pH value) − (control basal pH value [~4.8]). The control basal pH value was obtained by using cells loaded with FD-70 as described above. These were handled exactly like cells used in experimental recordings, except that cells were not exposed to CFPE or any other specific protein. Recordings were referred to the calibration curve (Fig. 1) to obtain corresponding pH values which were averaged over a 90-min recording period.

For the calibration curve (Fig. 1), results are expressed as the ratio of 550-nm emission intensities at excitation wavelengths of 495 and 450 nm: I_{495}/I_{450} = (intensity emission at 550 nm resulting from a 495-nm excitation)/(intensity emission at 550 nm resulting from a 450-nm excitation).

RESULTS

Effect of *M. tuberculosis* CFPE on J774 macrophage lysosomal pH. The incubation of FD-70-loaded macrophage cultures with 500 μg of CFPE per ml at 4°C for 5 min induced multiple alkalinization-acidification cycles in macrophage lysosomes (Fig. 2A). These alkalinization-acidification kinetics were determined with the aid of the calibration curve in Fig. 1. (Since fluorescein has a pH-dependent fluorescence excitation profile, the pH of fluorescein-containing organelles can be determined from the ratio of the fluorescence intensities resulting from 450- and 495-nm excitation [13].) The first cycle peaked 30 min after washing with PBS. It was a reversible process which reached an alkalinization maximum with respect to the baseline pH (~4.8) of more than 1 pH unit. A second peak could be observed 60 min after washing. As with the first one, it was reversible and of comparable magnitude.

The experiment shown in Fig. 2A was repeated with the mycobacterial antigens present throughout the complete 90-min monitoring period to test whether a synchronization of initial antigen internalization is a prerequisite for the alkalinization-acidification cycles observed. The use of this prolonged incubation period resulted in similar cyclic kinetics and pH maxima (Fig. 2A and B). Preliminary results (data not shown) indicated similar behavior for CFPE obtained from the avirulent strain *M. tuberculosis* H37Ra.

When the antigen filtrate was heated for 30 min at 56°C, the lysosome alkalinization magnitude was significantly reduced (Fig. 2D). A residual alkalinization of about the same magnitude (pH of <0.5) was observed when macrophages were incubated with equivalent amounts of other proteins such as bovine serum albumin and transferrin (Fig. 3).

Effect of *M. tuberculosis* CFPE on P388D.1 macrophage lysosomal pH. Exposure of P388D.1 cells to 500 μg of CFPE per ml for 5 min at 4°C induced an alkalinization effect (Fig. 2C) similar to that seen for J774A.1 cells (Fig. 2A).

Alkalinization dependence on CFPE concentration. To test whether lysosome alkalinization is a saturable process we recorded alkalinization kinetics and maxima for several CFPE concentrations. Alkalinization kinetics were not significantly different within a range of 100 to 1,000 μg of CFPE

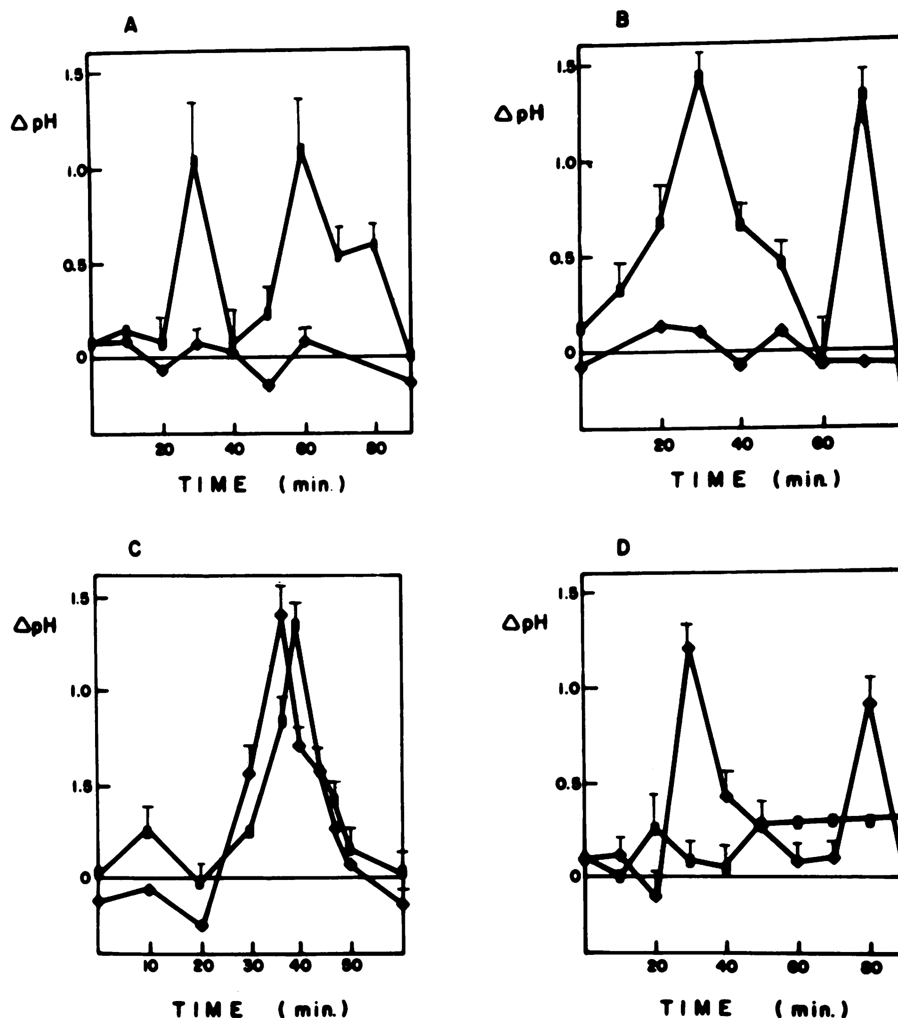

FIG. 2. Alkalinization kinetics of macrophagelike cell lysosomes induced by CFPE. (A) J774A.1 cells preincubated for 5 min at 4°C with CFPE (■), control cells (◇); (B) J774A.1 cells incubated for 90 min at 37°C with CFPE (■), control cells (◇); (C) cells preincubated as in A, P388D.1 cells (◇), J774A.1 cells (■); (D) J774A.1 cells preincubated as in A (◇), cells preincubated with CFPE heated at 56°C for 30 min (■). Results are expressed as means + standard errors.

per ml (results not shown); however, maximum lysosomal alkalinization increased as a function of concentration throughout this same range (Fig. 4), reaching an apparent saturation level at around 700 to 1,000 μg/ml. Using ^{125}I-labeled antigens and assuming an average antigen molecular mass of approximately 50 kilodaltons and individual antigen fractional concentrations of under 10% (14), we have determined an upper limit of 10^7 mycobacterial antigen molecules per cell as the actual concentration of intracellular effectors in our experiments.

DISCUSSION

These experiments show that the addition of *M. tuberculosis* CFPE to mouse-derived macrophagelike cells induces a reversible alkalinization, which is concentration dependent and saturable and exhibits cyclic behavior in the J774A.1 lysosomal lumen. Cyclic kinetics were confirmed by results obtained from a prolonged incubation of the cells with CFPE (Fig. 2B), in which a synchronization of initial antigen internalization was shown not to be required for the alkalinization-acidification cycles. The first cycle detected in the J774A.1 cells was also found in the P388D.1 cell line, which has a different lysosomal enzyme composition (17), suggesting that this cyclic process may be found in different cell lines independently of their lysosomal enzyme concentrations. A more detailed kinetic study would be required to characterize the periodicity of these cycles. Finally, the observation that neither heat-inactivated mycobacterial antigens nor proteins such as albumin or transferrin were able to induce alkalinization-acidification cycles in these lysosomes (Fig. 2D and 3) suggests that one or more heat-labile antigenic components are required for the results reported here.

A plausible explanation for the results obtained would require the existence of a mycobacterial antigen(s) capable of disrupting the function of some lysosomal structure essential for lysosomal acidification (membrane permeability, H^+-ATPase pump, proton chemical activity in the lumen). The fact that the second alkalinization cycle begins only after the initial pH has been regenerated suggests a molecular mechanism which is pH dependent. This cyclic mechanism has been widely reported for toxin-mediated entry and

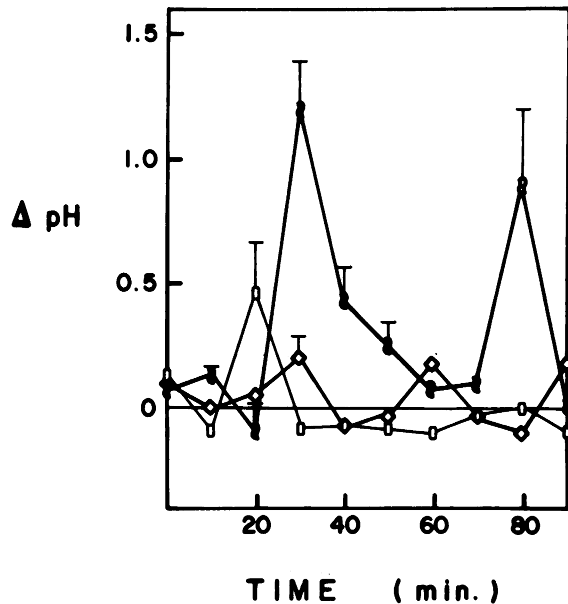

FIG. 3. Effects of other proteins on the lysosomal pH of J774A.1 cells. Cells were preincubated with BSA (◇), transferrin (■), or CFPE ( ). Results are expressed as the means + standard errors.

damage to host cells by other kinds of intracellular parasites (4, 12, 19).

Acidification of intracellular compartments has also been suggested for the process of fusion between these organelles (9). Among the possible consequences of alkalinization of the lysosomal lumen, we could have a decrease in the hydrolytic activity of its enzymes and an impairment of fusogenic activity (9). This could explain the pulselike behavior observed, since only when the agent has been metabolically inactivated (by lysosomal degradation), such that the normal lysosomal pH value can be regenerated, can a second pinocytic vesicular antigen load fuse with the secondary lysosomes and induce a new alkalinization cycle. The fact that pinocytic vesicles do not seem to be affected in their fusogenic activity (alkalinization is measured in the lysosomes) suggests that this alkalinization requires a minimum pH threshold between the baseline value for lysosomes

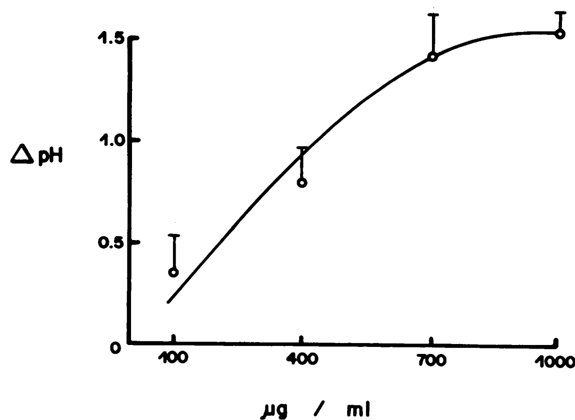

FIG. 4. Dependence of alkalinization on CFPE concentration.

(4.5 to 5.0) and pinosomes (6.0) for its triggering. Threshold pH values for endosomal membrane insertion have been reported in other systems, such as Semliki Forest virus and tetanus toxin (4, 12).

The inhibition of phagosome-lysosome fusion (1) mediated by a direct effect on lysosomes (5, 7) rather than on phagosomes (10, 15) would be a probable consequence of the observed alkalinization. The fact that other observations (2) have indicated that even when phagosome-lysosome fusion is not inhibited many internalized mycobacteria do survive supports the idea of a direct effect on lysosomal function of an alkalinizing agent.

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