CHEMICAL INHIBITORS OF PHAGOSOME-LYSOSOME FUSION IN CULTURED MACROPHAGES ALSO INHIBIT SALTATORY LYSOSOMAL MOVEMENTS

A Combined Microscopic and Computer Study

BY P. D'ARCY HART,* M. R. YOUNG,*‡ M. M. JORDAN,§ W. J. PERKINS,§ AND M. J. GEISOW‖

From the Laboratory for Leprosy and Mycobacterial Research, the Computing Laboratory, and the Biophysics Division, National Institute for Medical Research, London NW7 1AA, United Kingdom

Lysosomal movements could be of critical importance for successful phagosome-lysosome (P-L)[1] fusion within phagocytes. Agents affecting fusion might operate either directly upon the fusion process or by altering the movements of lysosomes and so the frequency of their contact and fusion with the phagosomes. A number of chemical agents (polyanions, certain weak bases, and phorbol myristate acetate) have been previously reported to modulate P-L fusion (inhibiting or stimulating) in cultured macrophages (1–11). We now report that the fusion inhibitors ammonium chloride (NH_4Cl), a weak base, and poly-α-D-glutamic acid (PGA), a representative polyanion of low toxicity, also inhibit saltatory lysosomal movements. Direct microscopic examination is supported by computer analysis techniques. Such alteration of lysosomal movement could explain the effects of these inhibitors of P-L fusion without involving direct action on the fusion process itself.

Materials and Methods

Compounds. PGA (sodium salt, mol wt ~74,000) was obtained from Sigma Chemical Co, St. Louis, MO, ammonium choride from British Drug Houses, Ltd., Poole, Dorset, UK, suramin from ICI Pharmaceuticals, dextran sulphate (average mol wt 2×10^6) from Pharmacia Fine Chemicals, Piscataway, NJ.

Cell Cultures. Resident mouse peritoneal macrophages from female P strain mice were established as monolayers in Chang medium (containing 40% horse serum) on coverslips in Leighton tubes for 1–2 wk (12), with a medium change if extended beyond 9 d. The time in culture and the maturity of the cells were important: lysosomal movements in cells that were kept in culture for 5 d or less were sometimes difficult to observe because the cells were not yet fully extended, while old cells (4 wk or more in culture) tended to show reduced lysosomal movements and P-L fusion responses. Unless stated otherwise, treatments of the macrophages with chemical agents and other manipulations and assays were carried out on the monolayers in Hanks' balanced salt solution (HBSS) after washing with

* Laboratory for Leprosy and Mycobacterial Research.
‡ Recipient of a grant from the Royal Society.
§ Computing Laboratory.
‖ Biophysics Division.

[1] *Abbreviations used in this paper:* AO, acridine orange; HBSS, Hanks' balanced salt solution; PGA, poly-α-D-glutamic acid; P-L, phagosome-lysosome.

that solution; treatments extending overnight or more were carried out in Chang medium. The lengths of treatments were related to the rates of uptake of the agents by the macrophages (for details of procedures see protocols to Tables I and IV and Results).

Assessment of Lysosomal Movements by Direct Microscopy. Coverslips were mounted in an observation chamber, transferred to a microscope incubator (37°C), allowed 5–10 min to achieve culture temperature, and surveyed under a 100× oil-immersion phase-contrast objective. The source of illumination was a tungsten filament with heat filter and a narrow band Kodak Wratten No. 61 green filter. Direct visual microscopy was used routinely to assess movements of the lysosomes, which form the main population of vesicles surrounding the nucleus. This procedure permitted a qualitative assessment based on a detailed examination of cells surveyed randomly over the monolayer area, and could be carried out rapidly for many monolayers. The degree of movement was assigned to one of five grades (See Table I, footnote).

Fluorescence Assay of P-L Fusion. Transfer of free fluorescent acridine orange (AO) label from secondary lysosomes to phagosomes was assayed by a system previously described (1, 3, 7). Live commercial *Saccharomyces cerevisiae* (10^6–10^7 yeast cells/ml) were offered for ingestion by macrophages in monolayers at 37°C, either continuously or as a 15-min pulse followed by washing and reincubation. At 50 min or more after the start of ingestion, the macrophages were examined by fluorescence microscopy for P-L fusion. The macrophages had been previously labeled for 10 min at 37°C with 5 μg/ml AO and washed. When, however, assessment of P-L fusion was to be combined with assessment of lysosomal movements in the same cells, the labeling was varied to avoid the presence of AO in the lysosomes during phase microscopy and the consequent risk of photosensitization (13); the labeling with AO was then done as a final procedure and fluorescence microscopy performed 5 min later (7). The presence within phagosomes of confluent "rims" of orange fluorescence surrounding the enclosed yeasts, or of subsequent staining of the yeasts, was regarded as positive for P-L fusion (1); the method is semiquantitative, giving an overall evaluation (8).

Critique of the AO Assay. The inhibition of P-L fusion by polyanions (PGA, dextran sulphate, etc.) and by NH_4Cl was first recognized by the AO assay. Inhibition by polyanions has been corroborated using a fluorescent polyanion instead of free AO to label lysosomes (6), as well as by electron microscopy (with ferritin or thorotrast as a marker) (1–4, 8–10). Inhibition by NH_4Cl has been confirmed by substituting fluorescent dextran for AO as a lysosomal marker (unpublished observation) and also by electron microscopy (7).

Nevertheless, weak bases that are known to reduce lysosomal uptake of AO (14, 15) and to increase intralysosomal pH (16) might interfere with the P-L fusion assay by causing a visible decrease in lysosomal staining by the dye (17). We confirm that such a decrease may occur after NH_4Cl treatment of macrophages, but that by labeling initially (pulse before exposure to the NH_4Cl) or terminally (after the yeast incubation) this decrease has been no more than slight and the orange/red color has been retained.

To test whether a change of this order is likely to have affected our AO assays of P-L fusion, we incubated yeast-containing normal (untreated) macrophage monolayers for 50 min, then assayed P-L fusion after adding AO in graded concentrations to the medium (HBSS), ranging from the standard 5 to 0.3 μg/ml. A normal fusion score was retained until the red or orange staining of nonfused lysosomes was replaced by yellow, estimated to correspond to an intralysosomal AO concentration of about ⅛ or less of the standard, well below that encountered by us after treatment with NH_4Cl (5 or 10 mM). We conclude that under our experimental conditions, any effect of this base on AO lysosomal staining is insufficient to have diminished an appreciation of the transfer of the dye to phagosomes and consequently to have overestimated inhibition of P-L fusion. The use of fresh yeasts, together with the two-stage fusion assay, may have contributed to reliability.

The adequacy of the AO technique for the present purposes is supported by two further considerations. First, corresponding to the formation of phagolysosomes by P-L fusion, degranulation (loss of lysosomes) becomes evident after some hours, as assessed by labeling the remaining lysosomes with AO as a terminal procedure immediately before examination. Degranulation was much reduced by NH_4Cl treatment of the macrophages

(Fig. 1 and reference 7), and the large number of nonfused lysosomes recognized as remaining in the cytoplasm excludes the possibility that a fusion response had been missed and the AO appearances wrongly attributed to inhibition. Second, chloroquine and some other lipophilic secondary and tertiary amines are also weak bases and have similar effects to NH_4Cl on intralysosomal pH and, potentially, lysosomal AO staining and sequestration (17). After treatment with these amines, transfer of the dye is plainly visible, and its frequency and extent even exceeds that in normal cells, indicating enhancement of P-L fusion (3, 4, 8); the enhancement is corroborated by other assays of P-L fusion (3, 4, 6, 8).

Assessment of Lysosomal Movements by Computer Methods. Sequential photo flash (0.5 ms) 35-mm micrographs, taken at intervals of 2, 10, and 30 s, were used in the computer assessment on the assumption that after such intervals, lysosomes in normal cells would have changed their position many times while those in drug-treated cells, if static, would not show a comparable change. (Individual lysosomes in normal cells can move in a saltatory manner up to several times a second, and in the present experiments the path length of the movements was estimated as 1–2 μm.) Between each flash exposure at the 10- or 30-s intervals, the image was refocused if necessary. At the intervals chosen, preliminary experiments showed that the observations were not appreciably affected by the continuous but much slower movement of the whole cell as it changed shape.

These serial micrographs provided the data for the assessment of lysosomal movement by two computer techniques. In the first, use was made of an interactive graphics system (18) to provide a comparative display of lysosomal movement over a chosen time interval after different treatments ("graphics assessment"). The second was a quantitative analysis using a "nearest neighbour" technique that paired lysosomes at different times on the basis of proximity and could be used as an estimate of lack of movement.

Two series of cells were sampled from different macrophage monolayers that had been given one of the following treatments: (*a*) NH_4Cl 10 mM in HBSS at 37°C for 30–40 min; (*b*) PGA 100 μg/ml for 5 d in culture medium; or (*c*) no treatment (normal). In series 1 the cells were selected as typical of the treatment being studied after visually monitoring the parent monolayers for lysosomal movements and (after photography) for P-L fusion, using phase and fluorescent microscopy, respectively. In series 2 the cells were randomly sampled. In series 1, two cells were studied for each treatment, one at 0 and 2 s, the other at 10-s intervals from 0 to 20 or 30 s. In series 2, two cells were again studied for each treatment, one at 0 and 2 s and the other at 0 and 30 s. Enlarged prints of the photomicrographs (final magnification × 2,200) were covered with a transparent plastic sheet on which the positions of the cell boundaries and all visible lysosomes were traced. Fixed reference markers, which remained stationary during the recording time and could be easily recognized, were also indicated in each tracing for use in later alignment of the cells over an interval; these markers consisted of three or more well separated features usually inside the cell being studied, such as prominent fat droplets. All tracings were made by the same person to ensure that the variability in any error introduced at this stage was minimal.

Transfer of Data to Computer. The coordinates of each cell boundary, lysosome, and reference marker were entered manually into a PDP 11/34 computer using a graphics tablet (19). Each cell at the two times being compared was aligned as closely as possible before entry, by means of the reference markers alone. The outline of the cell boundary was entered by tracing over it with the tablet probe and the positions of the lysosomes and reference markers were recorded as coordinate pairs (x, y) by touching them with the probe. In repeated recordings of the positions of each lysosome, the error was $\sim\pm$ 0.2 mm on the enlarged print of the original photomicrograph (equivalent to $\sim$0.09 μm).

Graphics Assessment. The computer software then made it possible to display any cell, showing the margin and indicating reference markers and lysosomes with specific symbols and colors. The patterns for the same cell at two (or more) successive intervals could also be superimposed to provide a visual appreciation of relative movement over that period. Also the alignment of reference markers could be checked and the cells realigned if necessary. In all cases the cell boundaries in superimposed patterns matched well, giving

similar values for boundary lengths and cell areas over the time intervals considered. Cell boundary movement was therefore not thought to affect the assessment of lysosomal movements in these cells to an appreciable extent.

The colors used in the display of the lysosomes at two times were red and green so that if any lysosomes overlapped they appeared yellow. Movement of the lysosomes leading to less overlap should thus produce a display with predominantly red and green and little yellow, whereas lack of movement should show overlap and a significant amount of yellow.

Nearest Neighbour Technique. Since individual lysosomes at the different times could not easily be related to each other on a one-to-one basis in terms of position, it was assumed that the lysosomes closest to each other could be treated as corresponding pairs. A computer algorithm was devised to pair lysosomes that were nearest neighbors when the lysosomes at time 0 were compared with those at time 1 (2, 10, 20, or 30 s). As part of the algorithm, the user could supply a recognition distance within which two lysosomes at different times must lie for them to be considered a pair. In every case, the closest lysosomes were taken as the corresponding pair even if several lysosomes at time 1 lay within the given recognition distance from a time 0 lysosome.

The result of the algorithm was the calculation of the number of corresponding pairs and the total number of lysosomes recorded in the cell at time 0 and time 1 (2, 10, 20, or 30 s). This analysis was performed for all the lysosomes identified in the cells to obtain sufficient numbers for reliable results.

Results

Effects of Inhibitors of P-L Fusion on Lysosomal Movements Studied by Phase Microscopy. Direct visual phase microscopy of untreated (control) monolayer macrophages in HBSS showed characteristic intracellular (cytoplasmic) undulatory movements (20) and non-Brownian, saltatory lysosomal movements (21–25). Moving particles were recognized as lysosomes by their number, size, and appearance (cells cultivated in medium with high serum content develop particularly conspicuous lysosomes [26]), confirmed by their AO fluorescence and previously reported correlation with acid phosphatase cytochemistry (27). The usual substantial degree of P-L fusion was noted after ingestion of yeasts (1).

We have previously reported that NH_4Cl inhibits P-L fusion in mouse macrophages (7); the effect is illustrated in Fig. 1. We have now observed, in monolayers examined by phase microscopy after treatment with 5 or 10 mM NH_4Cl in HBSS at 37°C for 0.5–2 h (varying with different cell series), a "paralysis" of the lysosomal movements (Table I). With 10 mM NH_4Cl the lysosomes were stationary in ~90% of cells; with 5 mM the stasis varied from 90 to 70% and movements were sluggish in the remainder. After phagocytosis of yeasts, NH_4Cl-treated cells displayed continuing lysosomal stasis, and subsequent AO labeling demonstrated the expected inhibition of P-L fusion (Table I). The inhibitory effects of NH_4Cl (5 mM) on both movement and fusion began to fade after 4–5 h of continued exposure at 37°C. Methylamine (as hydrochloride), a weak inhibitor of P-L fusion (7), was also a weak inhibitor of lysosomal movements; the lowest concentration to produce an effect was ~10 times that of NH_4Cl (5–10 mM compared with 0.5–1.0 mM).

The polyanionic inhibitor of P-L fusion, PGA (3, 4, 8–10), was also observed (at 100 μg/ml) to inhibit lysosomal movements markedly, although compared with the results of NH_4Cl treatment stasis occurred less uniformly and uptake was slower, requiring 5 d of incubation for maximal effect (Table I). Movements were also inhibited by the polyanions suramin (100 μg/ml) and dextran sulphate

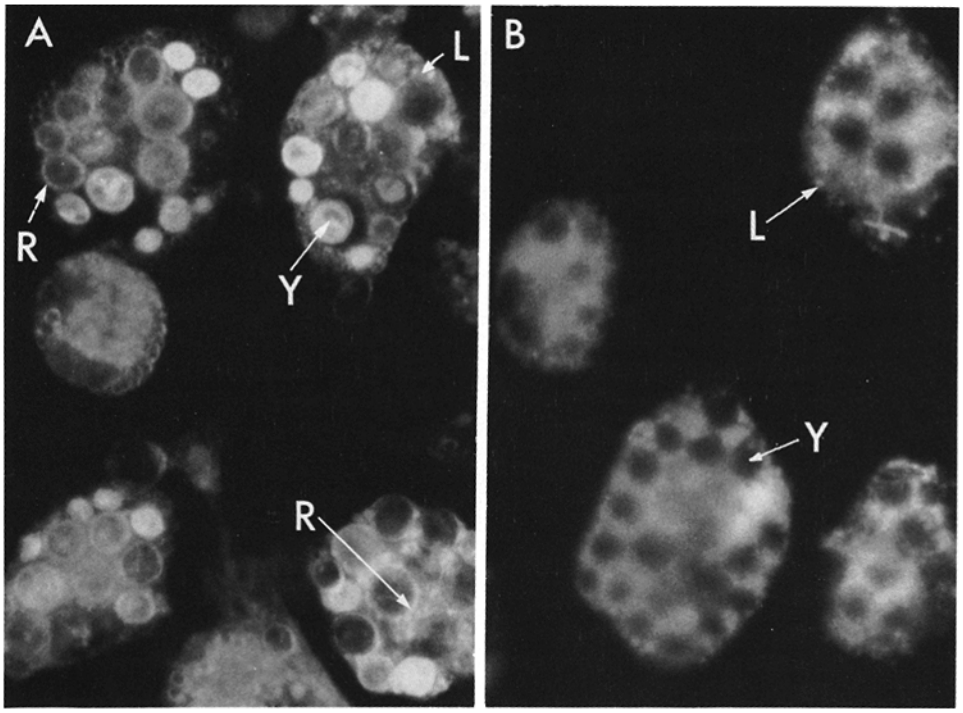

FIGURE 1. AO fluorescence assay of P-L fusion and its inhibition by ammonium chloride, in macrophages after ingestion of live *Saccharomyces cerevisiae*. (*A*) Untreated cells. Confluent "rims" (R) of orange fluorescence surrounded yeasts (Y) within phagosomes; many yeasts fluoresced green or red. Evident degranulation, with few nonfused lysosomes (L). Positive fusion response. (*B*) NH_4Cl-treated. Most intraphagosomal yeasts (Y) were unstained (dark spaces), and cytoplasm was packed with orange-red nonfused lysosomes (L). Nonfusion response. (The appearances would be similar in PGA-treated cells). From color micrographs; × 1,300.

(10 μg/ml), each after 3 d of incubation (data not shown); these compounds are known inhibitors of P-L fusion (1, 4).

Macrophages treated with any of these agents appeared healthy and showed phagocytic indices for yeasts similar to those shown by normal controls. Lysosomal swelling was variable but generally slight or moderate.

Computer Assessment and Analysis of the Inhibition of Lysosomal Movements. Each of the two series studied by computer techniques was composed of NH_4Cl-treated, PGA-treated, and untreated (normal) cells, either selected as typical of the treatment effects (series 1) or randomly sampled (series 2) (see Materials and Methods).

Graphics Assessment. Changes in lysosome position with time in each cell in the two series were examined using the computer color display of red/green/yellow lysosomes to assess lack of movement (see Materials and Methods). Cells treated with NH_4Cl or PGA showed considerable overlap in lysosome position and thus little movement; the normal cells showed little overlap, indicating considerable movement. An enlarged monochrome reproduction of plots of parts of superimposed cells from the 0–30 s group, series 2, is shown in Fig. 2,

TABLE I

Lysosome Movements and P-L Fusion in Macrophages Treated with the Fusion Inhibitors NH_4Cl and PGA (Direct Visual Observation)*

Treatment	Agent's fusion status	Change of movements from normal‡	Fusion§
			%
HBSS alone	Normal	0	70
NH_4Cl	Inhibitor (7)	−3	10
PGA	Inhibitor (3, 4, 8–10)	−2	10

* Monolayers pretreated at 37°C for 60 min with NH_4Cl (10 mM) in HBSS, or with HBSS alone (controls), were examined (at 37°C) for lysosomal movements by phase microscopy; they were then incubated with yeasts for 50 min (with the drug still present), labeled with AO, and assessed for P-L fusion by examining by fluorescence microscopy 5 min later (see Materials and Methods). Other monolayers were incubated for 5 d with PGA (100 μg/ml) in Chang medium or in medium alone; they were then washed and examined in HBSS for lysosomal movements and P-L fusion.

‡ Overall assessment of lysosome movements expressed as change from normal, on the following scale: −3, no movement; −2, none in most cells, sluggish in remainder; −1, slightly reduced movement; 0, normal movement; +1, hyperactive.

§ Scored as proportion of yeast-containing phagosomes showing intraphagosomal fluorescent rims or colored yeasts (see Materials and Methods).

with colors replaced by symbols. Since this reproduction is larger than that appearing on the display, closeness of the symbols replaces overlap as the criterion of stasis. It can be seen that the symbols are closely paired over the interval in the NH_4Cl-treated cell (Fig. 2*A*) but more widely dispersed in the normal cell (Fig. 2*B*).

Nearest Neighbor Technique. To obtain a quantitative assessment of the levels of correspondence in lysosome position over the different time periods, a "nearest neighbor" procedure was used (see Materials and Methods). In series 1, comparison was made between 0 and 2 s in the first group of cells, and between 0 and 10, 10 and 20, and 20 and 30 s in the second group. In series 2, the comparisons were made at 0 and 2 s in one group of cells and at 0 and 30 s in the other. The procedure was repeated in each case for several recognition distances from 1 to 3 mm (~0.5–1.5 μm actual) in steps of 0.5 mm. Distances of <1 mm were not considered because the error from transferring the data to the computer could be of this order. Increasing the recognition distance increased the likelihood of pairing. A large recognition distance, however, increased the likelihood of pairing lysosomes that did not correspond. The maximum distance considered therefore was 3 mm.

Fig. 3 shows the results of this analysis for the series 1, 0–2 s group, with the corresponding pairs shown as a percentage of the mean number of lysosomes between time 0 and time *t*. (Since the NH_4Cl-treated and PGA-treated cells were studied on different occasions, each had its own normal untreated control cell.) The differences in pairing between the drug-treated cells and their normal control cells were tested at each recognition distance using a chi-squared test. In

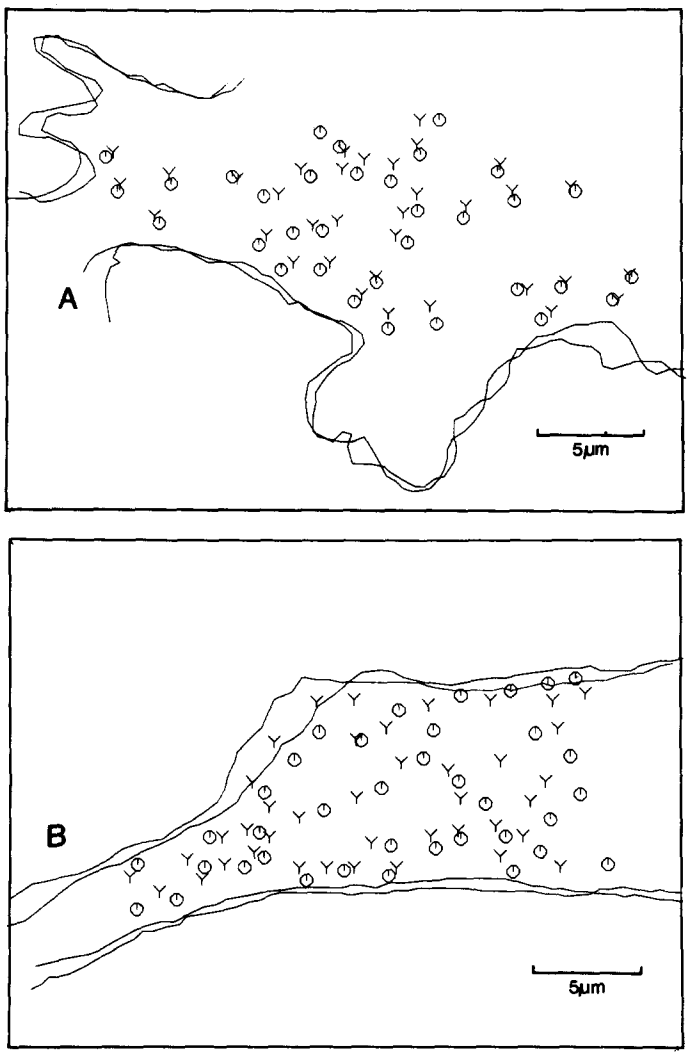

FIGURE 2. Computer plots of lysosomes at 0 s (Ⓨ) and 30 s (Y) in a cell treated with NH_4Cl (*A*) and in an untreated cell (*B*). It is possible to pair most of the lysosomes in *A* but not in *B*, indicating more movement over the time period in the untreated cell.

the following results, the significance level applies to all the recognition distances unless stated otherwise. It can be seen in Fig. 3 that both the NH_4Cl cell and the PGA cell show much larger percentages of corresponding pairs than do the untreated normal cells, indicating less movement in the former ($P < 0.001$). Fig. 4 shows, also for series 1, the data taken at 10 s intervals (data for the NH_4Cl-treated cell were only available up to 20 s for technical reasons). The NH_4Cl-treated cell again shows a much higher percentage of corresponding pairs at each 10 s interval than does the normal cell (Fig. 4*A*) ($P < 0.001$). The PGA cell was less consistent; it shows much higher pairing than the normal cell at 0–10 and 20–30 s ($P < 0.001$), but only a small though significantly higher pairing at 10–20 s ($P < 0.05$) (Fig. 4*B*).

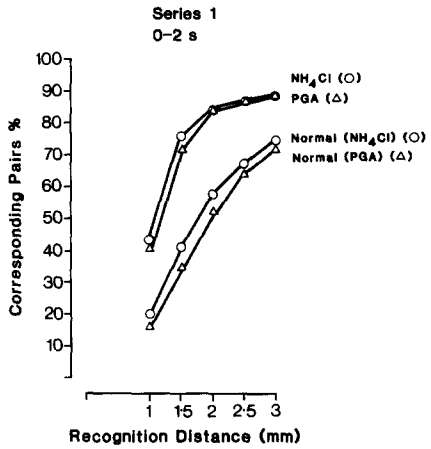

FIGURE 3. Paired lysosomes over 2-s intervals (series 1) expressed as a percentage of the average number of lysosomes over the period. The results are shown for different recognition distances and show much higher pairing in the NH_4Cl- and PGA-treated cells than in the untreated (normal) cells. High pairing indicates lack of movement over the interval. The NH_4Cl and PGA cells each have their own normal control since the experiments were performed at different times.

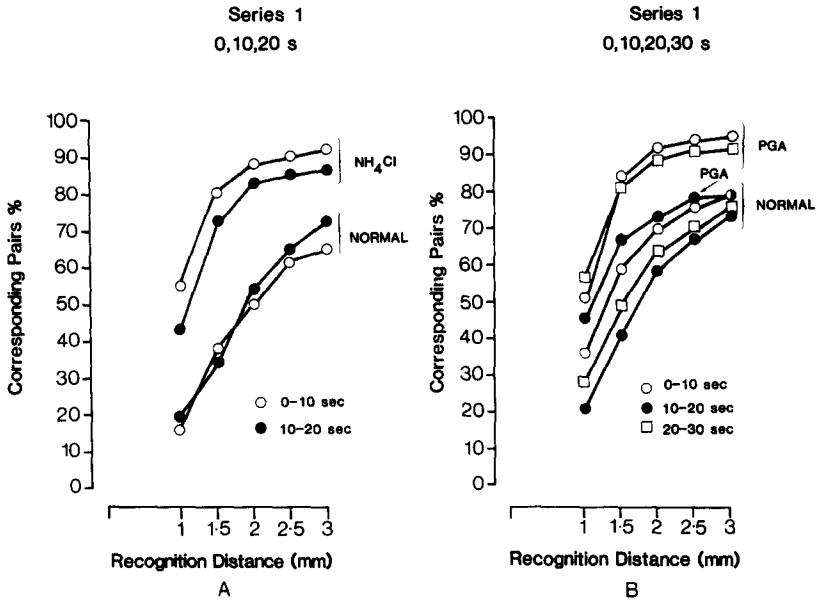

FIGURE 4. Paired lysosomes over 10-s intervals (series 1) expressed as a percentage of average number over the interval and shown for different recognition distances. (*A*) The NH_4Cl-treated cell at 0–10 and 10–20 s; (*B*) the PGA-treated cell at 0–10, 10–20, 20–30 s. In both *A* and *B*, the results are compared with a corresponding normal untreated cell photographed at the same time.

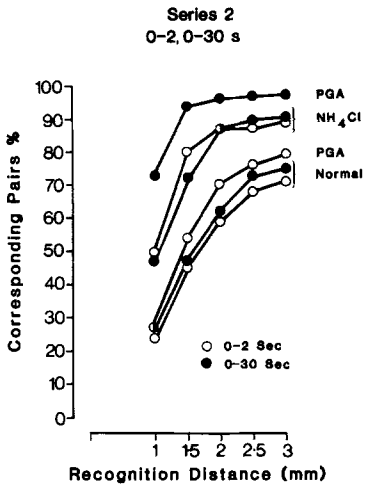

FIGURE 5. Paired lysosomes for two separate groups of cells (2-s and 30-s intervals) in series 2 expressed as a percentage of the average number of lysosomes over the interval and shown for different recognition distances. The PGA and NH_4Cl-treated cells are compared with the same normal untreated cell at 0–2 s and similarly at 0–30 s.

The results for series 2 (Fig. 5) are similar to those for series 1. Again the NH_4Cl-treated cell (0–2 s and 0–30 s) shows consistently much higher pairing than the normal cell ($P < 0.001$). The PGA-treated cell shows much higher pairing than the normal cell in the 0–30 s group ($P < 0.001$), but the difference is small in the 0–2 s group ($P < 0.05$ at recognition distances 1.5, 2, and 3 mm, and not significant at other distances).

Taking an overall view of all the information from the two series, all the NH_4Cl-treated cells showed a similar pattern with very high pairings (e.g., 80–90% at a recognition distance of 2 mm [1 μm actual distance] and ~90% at 3 mm). The normal cells showed more variation, but the range of values was consistently lower than for the NH_4Cl-treated cells (50–70% at 2 mm and 65–78% at 3 mm). The PGA-treated cells generally showed very high pairings (80–96% at 2 mm and 90% or more at 3 mm), but on a few occasions they showed values only just above the upper end of the range of the normal cells. The percentage pairings in the normal cells may seem high but this is due to two factors; first, the active lysosomes move within a restricted area and may return to a point near their original positions; second, the technique searches up to 3 mm (1.5 μm actual distance) from the original position and a pairing at this distance may be made with a lysosome that does not correspond.

The method used thus far only considered movement in the X-Y plane, i.e., the focal plane of the microscope, but if lysosomes are moving they will also move in the Z plane, i.e., in and out of focus. Since the depth of field was of the order of only 0.25 μm, which is approximately the diameter of lysosomes under the conditions of these experiments, appreciable movement in the Z plane, with consequent changes in the number of lysosomes observed over the time period, would be expected. Such changes should be particularly noticeable in a cell with active lysosomes. Tables II and III show for the two series the numbers of

TABLE II

Comparison of the Numbers of Lysosomes in Series 1 Cells for Different Treatments at the Intervals Shown (Data Used in Computer Analysis)

Time interval	NH_4Cl	Normal	PGA	Normal
0–2 s				
Number at 0 s	187	173	312	194
Number at 2 s	186	196	303	205
Difference*	1	23	9	11
Percent difference‡	0.5	12	3	6
0–10 s				
Number at 0 s	208	194	316	314
Number at 10 s	215	165	308	310
Difference	7	29	8	4
Percent difference	3	16	3	1
10–20 s				
Number at 10 s	215	165	308	310
Number at 20 s	221	147	283	246
Difference	6	18	25	64
Percent difference	3	12	8	23
20–30 s				
Number at 20 s	—§	—	283	246
Number at 30 s	—	—	298	250
Difference	—	—	15	4
Percent difference	—	—	5	1

There are two groups of cells, one studied over 0–2 s and the other at 10-s intervals from 0 to 20 s (NH_4Cl) or 0 to 30 s (PGA).

* Difference in number over the interval.

‡ Difference expressed as a percentage of the mean number of lysosomes over the interval.

§ Not done.

TABLE III

Comparison of the Numbers of Lysosomes in Series 2 Cells for Different Treatments over the Intervals Shown (Data Used in Computer Analysis)

Time interval	NH_4Cl	PGA	Normal
0–2 s			
Number at 0 s	170	225	214
Number at 2 s	171	226	239
Difference*	1	1	25
Percent difference‡	0.6	0.4	11
0–30 s			
Number at 0 s	100	243	171
Number at 30 s	99	245	156
Difference	1	2	15
Percent difference	1	0.8	9

There are two groups of cells, one studied over 0–2 s and the other over 0–30 s.

* Difference in number over the interval.

‡ Difference expressed as a percentage of the mean number of lysosomes over the interval.

lysosomes recorded in each cell at each time, the difference between each time, and this difference expressed as a percentage of the mean number of lysosomes over the time interval. In cells with active lysosomal movement in the X-Y plane, the change in numbers of lysosomes observed over an interval should be larger than in those with little or no movement. In Table II (series 1), the untreated cells show more change (up to 23%) than the NH_4Cl-treated cells (up to 3%) or the PGA-treated cells (up to 8%), supporting the previous results in Figs. 3 and 4. Table III shows, similarly, the changes in numbers of lysosomes over the time intervals for series 2. Again the normal untreated cells show more changes in number (up to 11%) than the NH_4Cl and PGA cells (up to 1% in both).

Restoration of Lysosomal Movements and P-L Fusion. Washed, ammonia-treated macrophages rapidly lose their fusion-inhibitory and other lysosomal responses to this base (7, 16), presumably due to its exit from these structures (28). We observed slowing of lysosomal movements as early as 5 min after exposure of monolayers to 10 mM NH_4Cl, with a maximal effect by 0.5–2 h, depending on the cells. Washing the monolayers followed by reincubation in plain HBSS rapidly reversed the stasis, restored normal movements, and, gradually, P-L fusion (Table IV *A*).

Analogy with Effect of Hypertonic Sucrose. Buckley (20) described the effects in cultured chick embryo cells of hypertonic sucrose, which stopped lysosomal saltatory movements within a minute or so of application. This stasis was associated with morphological changes in cytoplasmic filaments. It was reversed by washing. We tested hypertonic sucrose in macrophages to see whether it affected P-L fusion as well. Under the conditions used for NH_4Cl we confirmed that 0.2 M sucrose in HBSS rapidly suppresses lysosomal movements. Inhibition of fusion in the yeast system was now also observed, and both effects were rapidly reversed after washing (Table IV *B*). Moreover, if the sucrose treatment of the monolayers was prolonged to 18–48 h by incorporation in Chang culture medium, the inhibitory effects began to fade, with both functions partially restored.

Discussion

AO Assay of P-L Fusion. The transfer of AO from lysosomes to yeast-containing phagosomes has been used extensively to assay and to identify inhibitors and enhancers of P-L fusion (1–4, 6–9, 11), and the method has been used exclusively in the present study. We have considered some general problems of AO as a marker previously (8), and in some detail in Material and Methods. We have concluded that the technique is reliable for the types of experiment reported here.

Lysosomal Movements and P-L Fusion. The lysosomes of normal macrophages are in complex and continual (saltatory) motion (24). We have now observed that lysosomotropic agents, previously reported to inhibit P-L fusion (NH_4Cl and the polyanion PGA), also inhibit or suppress saltatory lysosomal movements. This inhibitory effect was shown also by two other tested anionic fusion inhibitors, namely suramin and dextran sulphate, and was confirmed for hypertonic sucrose (20), which was observed also to inhibit P-L fusion.

The computer analyses, both graphical and numerical (nearest neighbour technique), supported the observations obtained by visual microscopy, whether

TABLE IV

Reversal of Inhibition of Lysosome Movements and Phagosome-Lysosome Fusion by Washing with: (A) NH_4Cl, (B) Hypertonic Sucrose‡ (Direct Visual Observation)*

(A)

Time	Washed				Not washed	
	HBSS alone		NH_4Cl		NH_4Cl	
	Change of movements from normal§	Fusion‖	Change of movements from normal	Fusion	Change of movements from normal	Fusion
		%		%		%
Before wash	0	70	−3	5	−3	5
20 min after	0	70	−1	50	−2	5
2 h after	0	70	0	70	−2	10

(B)

Time	Washed				Not washed	
	HBSS alone		Sucrose		Sucrose	
	Change of movements from normal§	Fusion‖	Change of movements from normal	Fusion	Change of movements from normal	Fusion
		%		%		%
Before wash	0	75	−3	35	−3	35
20 min after	0	75	0	75	—¶	—
60 min after	0	75	—	—	−3	30

* Monolayers, pretreated at 37°C with NH_4Cl (10 mM in HBSS) for 60 min, were sampled immediately (at 37°C) for lysosomal movements by phase microscopy; the remainder continued incubation, either in plain HBSS after thorough washing with the latter, or left unwashed (i.e., still in NH_4Cl); at stated times they also were examined for lysosomal movements. Control macrophages were similarly processed, but in HBSS alone throughout. In all cases the examination for lysosomal movements was followed by exposure to yeasts at 37°C for 50 min, labeling with AO, and assessment of P-L fusion.

‡ Procedures similar to *A* but initial treatment was with sucrose (0.2 M in HBSS) for 5 min.

§ See Table I, footnote(‡), for scoring scale.

‖ See Table 1, footnote(§).

¶ Not done.

the cells used in these analyses were selected as typical (by visual microscopy) or were randomly sampled. The computer techniques were specially developed to study movements of large numbers of particles (manuscript in preparation). The graphic displays indicated, and the nearest neighbour analysis and statistical results confirmed, the inhibitory effects of NH_4Cl and PGA on lysosomal movements. The NH_4Cl-treated cells, however, showed more consistent results than the PGA-treated cells, again agreeing with the findings obtained by direct observation.

The lysosomal stasis in NH_4Cl-treated macrophages was reversed and P-L fusion restored by washing the monolayers and reincubating without the agent. Inhibition by hypertonic sucrose, both of lysosomal movements and P-L fusion, was also readily reversible.

The evidence presented here thus indicates an association between the two perturbed macrophage functions (lysosomal motion and P-L fusion) produced by the chemical agents under study. A more difficult question is whether change in motion and in fusion are independent effects or causally related. The latter alternative seems to provide the more rational concept, with a change in saltatory movements producing in turn a corresponding change in the frequency of periphagosomal assembly and ensuing P-L fusion. The effect of the agents on the fusion process would then be indirect. This could be the explanation even if the agents exercised effects on the lysosomal membrane, and potentially on its fusibility (as they, or at least the polyanions, probably do [29]); it is difficult to conceive that such membrane-mediated activity is realized at the phagosomal membrane when lysosomal movement is inhibited or suppressed and periphagosomal assembly thereby reduced or prevented.

Neither the inhibition of P-L fusion nor that of lysosomal movements by NH_4Cl is likely to have arisen from its basic property, since chloroquine, also a weak base, did not inhibit. Indeed, after treatment of macrophages with chloroquine diphosphate (30–100 μM for 60 min), the lysosomal movements were usually increased, and this increase was associated with the (previously reported) enhancement of P-L fusion in frequency and extent by this compound (3, 4, 8). Moreover chloroquine, which reverses PGA-induced inhibition of fusion (3, 4, 8), was now observed (at 30 mM and within 60 min) also to reverse PGA-induced lysosomal stasis (unpublished observation).

Microtubules may be important in the control of the saltatory movements of organelles (20–25, 30). In some (unpublished) experiments we observed that nonsaltatory lysosomal movements, appearing after microtubule deprivation of the macrophages (using colchicine or a cell-free phagosome-lysosome system [31]), did not show the slowing usually produced by NH_4Cl or PGA, suggesting that in normal cells microtubules may be involved in transmitting the modulating action of these agents. There is increasing evidence of a physical association of microtubule elements with lysosomal granules (e.g., 30). We suggest the possibility that the activity of the polyanions, which is known to be initiated in secondary lysosomes and probably in their membranes (6, 9, 29), is transmitted to associated microtubules that in turn direct and control the lysosomal saltatory movements. In such a mechanism, the effect of polyanions in increasing lysosomal membrane rigidity (29) might be involved. This problem is under investigation.

The present observations raise the question of whether pinosome-lysosome fusion is affected by inhibitors of P-L fusion. Kielian et al. (9), confirming that dextran sulphate inhibits P-L fusion in mouse macrophages, have reported that this polyanion does not inhibit pinosome-lysosome fusion in these cells. Uptake and intravacuolar distribution of fluid-phase pinocytic markers were unaltered when lysosomes contained this polyanion. We have made similar observations with NH_4Cl and PGA. Macrophages exposed to either 10 mM NH_4Cl for 30 min or to 100 μg PGA/ml for 5 d, and then to trypan blue (itself a polyanion), pinocytosed the dye sufficiently by 1 h at 37°C to show increased density in secondary lysosomes (assessed by phase microscopy), similar in degree to that in control cells also offered the dye; yet the PGA lysosomes appeared stationary during the pinocytic period. We suggest that the persistence of pinosome-

lysosome fusion is explained, in the present context, by the mobility of the pinosomes, which permits them to continue to make contact even with stationary lysosomes.

Summary

The effects on lysosomal movements produced by the weak base ammonium chloride and by a representative polyanion poly-D-glutamic acid (PGA), previously reported to inhibit phagosome-lysosome (P-L) fusion, have been studied in cultured mouse macrophages using direct visual phase-contrast microscopy, a previously described (1, 3, 7) fluorescence assay of fusion, and computer analysis techniques. Treatment of the macrophages with 5–10 mM NH_4Cl for 0.5–2 h or with 100 μg PGA/ml for 5 d caused a striking inhibition of saltatory lysosomal movements, as well as the expected inhibition of P-L fusion. Two other anionic fusion inhibitors tested, dextran sulphate and suramin, inhibited movements similarly. Removal of the NH_4Cl from the cell medium reversed the lysosomal stasis and restored P-L fusion.

Computer analyses of changes in lysosomal positions in treated and untreated macrophages during 2, 10, and 30-s intervals, using data from photomicrographs, computer graphics, and quantitative nearest-neighbour techniques developed for this purpose, supported the qualitative visual observation of the inhibition of lysosomal movements by the fusion inhibitors NH_4Cl and PGA. Over the chosen intervals, from 80 to 96% of the lysosomes could be paired within 1 μm of each other in the NH_4Cl- and PGA-treated cells in comparison with 50–70% in normal cells. The differences between the drug-treated and normal cells were highly significant.

In an analogous system, the lysosomal stasis induced by hypertonic sucrose was examined and it was observed that P-L fusion too was inhibited. Both effects were reversible.

We conclude that inhibition of P-L fusion and of lysosomal movement are associated. We suggest a causal relationship between these changes, namely, that the lysosomotropic inhibitors of fusion under study produce their effects largely, though perhaps not exclusively, by reducing saltatory lysosomal motion and consequently periphagosomal assembly, rather than directly and independently on P-L contact or on the fusion process itself. The possibility is raised that microtubules may be involved in the effector mechanism of these modulations.

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