Effects of cross-linked dimers of ribonuclease A or of lysozyme on the processing of endocytosed peroxidase by hepatoma cells

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Cross-linked dimers of ribonuclease, added at a concentration of 0.05 mg/ml to the culture medium of hepatoma (HTC) cells, were previously shown to inhibit intracellular degradation of peroxidase taken up by endocytosis. Intracellular localization showed that endocytosed peroxidase does not reach lysosomes in dimer-treated cells. The present study shows that preloading of lysosomes with fluorescent anti-peroxidase IgG, obtained by exposing HTC cells for 48 h to 0.1 mg of antibody/ml, restores intracellular degradation of endocytosed peroxidase. Moreover, accumulation of peroxidase into lysosomes, which no longer occurs in dimer-treated cells, occurs again under these conditions. We conclude that inhibition of transfer of peroxidase from phagosomes to lysosomes is most likely to be the alteration resulting from the exposure of the cells to ribonuclease dimer, rather than inhibition of fusion between phagosomes and lysosomes. The dimer of another basic protein, lysozyme, added at a concentration of 0.2 mg/ml to the culture medium, is shown to induce the same type of effects as does the dimer of ribonuclease; the half-life of endocytosed peroxidase increased from 5 to 15 h after 2 h exposure of HTC cells to dimerized lysozyme. The effect of both dimers on intracellular protein processing can be reversed by addition of 100 mM-galactose to the culture medium, up to 5 h after pretreatment of the cells. The dimers of ribonuclease A or of lysozyme have thus probably the same mechanism of action. Evidence that the two dimers share the same binding sites on the cells is presented.

Intracellular digestion of macromolecules taken up by endocytosis depends on the completion of several processes: interiorization of the substance in phagosomes, delivery of the phagosomal content to lysosomes and degradation by lysosomal hydrolases. Previous work in this laboratory (Bartholeyns *et al.*, 1979) has shown that the action of dimerized pancreatic ribonuclease upon rat hepatoma cells (HTC) in culture provided an experimental system in which digestion of horseradish peroxidase or of non-specific IgG taken up by these cells is inhibited. These macromolecules no longer accumulate in lysosomes, but remain associated with particles which have a lower equilibrium density in sucrose gradients. It could not be decided, however, if the effect of dimerized ribonuclease resulted from the absence of fusion between phagosomes and lysosomes or from the inhibition of transfer of the phagosomal content into the lysosomes.

Abbreviation used: IgG, immunoglobulin G.

The present study aimed to characterize further the defect in intracellular processing induced by dimerized ribonuclease in HTC cells. The reversibility of the phenomenon was also investigated and, since the action of the dimer does not appear to be related to its enzyme activity, the effect of another dimerized protein, lysozyme, was studied.

Materials and methods

Cell cultures

HTC cells derived from Morris hepatoma 7288C were cultured at 37°C in suspension in a glass spinner as described by Samuels & Tomkins (1970). The culture medium 213 (Gardner, 1969) was prepared by dissolving, per litre of double-distilled water: 9 g of Swim-77 powder, 0.5 g of NaHCO₃, 2 g of glucose; 5 mg of Phenol Red, 60 mg of penicillin G, 50 nmol of Tricine {*N*-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]glycine} and 5.5 mg of cysteine. After adjusting the pH to 7.85, the medium was

supplemented with 10% (v/v) foetal calf serum. The cells (initially 2×10^5 cells/ml) had a generation time of about 24 h and grew exponentially up to 1.2×10^6 cells/ml.

Fractionation

Homogenates (5×10^8 cells) were prepared in 0.25 M-sucrose, using 32 strokes of the tight pestle of a Dounce-type homogenizer (Blaessig Glass Specialities, Rochester, NY, U.S.A.).

Fractionation by density equilibration was performed in the automatic E40 titanium rotor assembly (Beaufay & Amar-Costesec, 1976) in a Beckman L5-50 centrifuge. The whole homogenate (10 ml) was layered on a 32 ml linear sucrose density gradient (1.08 – 1.27 g/cm³). To avoid formation of a pellet, a cushion of 6 ml of sucrose solution of 1.34 g/cm³ density was used. After centrifugation for 105 min at 45 000 rev./min ($\omega^2 \cdot dt$ 1.45×10^{11} rad² s⁻¹), 13 or 14 fractions were collected. Their densities were measured as described by Beaufay *et al.* (1974a) and were diluted in Triton X-100 (0.1 g/l) for analysis. Results are presented in the form of normalized histograms, according to de Duve (1967).

Assays

The methods used for the enzyme determinations can be found in the following references: neutral ribonuclease (EC 3.1.27.5), Bartholeyns & Baudhuin (1976); *N*-acetyl- β -glucosaminidase (EC 3.2.1.30), Leaback & Walker (1961), Libert *et al.* (1976); alkaline phosphodiesterase I (EC 3.1.4.1) and galactosyltransferase (EC 2.4.1.38), Beaufay *et al.* (1974a,b); peroxidase (EC 1.11.1.7), Strauss (1958); lysozyme (EC 3.2.1.17), Jolles (1960). Protein was determined by the method of Lowry *et al.* (1951).

The release of enzymes in the culture medium was studied in a serum-free medium in order to avoid interference from serum enzymes. Samples (5 ml) of culture medium were taken at different times and concentrated 50-fold on a Minicon sample concentrator (Amicon B.V., Oosterhout, The Netherlands).

For evaluation of the intracellular degradation of peroxidase, HTC cells were first cultured for 2 h in presence of horseradish peroxidase, then washed three times with culture medium, resuspended and further grown in fresh medium. Aliquots of cell suspension were taken every 1 h for peroxidase determination.

Morphological procedures

The techniques used for electron microscope examinations were those described by Leroy-Houyet *et al.* (1979). For cytochemical staining of peroxidase, cells were incubated for 8 h in a culture

medium including 1 mg of enzyme/ml. After fixation in 2% (w/v) glutaraldehyde and washing in 0.05 M-Tris/HCl buffer, pH 7.3, the cells were stained according to Graham & Karnovsky (1966) by incubation for 30 min at 37°C in the presence of 0.5 mg of diaminobenzidine/ml, 0.05 M-Tris/HCl buffer, pH 7.5, and 1% H₂O₂. Further processing for electron microscopy was performed as usual.

Materials

[³H]Acetic anhydride (50 mCi/mg) came from The Radiochemical Centre, ribonuclease A (type I-A), horseradish peroxidase, type II, egg-white lysozyme and yeast RNA type II-S from Sigma and dimethylsuberimide dihydrochloride from Pierce. Swim-77 powder, for the preparation of culture medium, and foetal calf serum were purchased from Gibco and ferritin from Serva. The synthetic dimer of ribonuclease was prepared by cross-linking with dimethylsuberimide dihydrochloride and was labelled as described previously (Bartholeyns & Baudhuin, 1976). For the dimerization of lysozyme, 50 mg of lysozyme was dissolved in 5 ml of 0.1 M-phosphate buffer, pH 8.5, and allowed to react for 2 h at 25°C with 3 mg of dimethylsuberimide; after gel-filtration on Sephadex G-75, the amount of dimer recovered was about 30%. The specific activity of dimer on *Micrococcus luteus* membranes was about 5-fold lower than that of the monomer.

Goat antiserum to peroxidase was purchased from Miles and anti-peroxidase IgG was purified by the method of Sober *et al.* (1956), followed by chromatography on a peroxidase-Sepharose 4B column; it was labelled with fluorescein isothiocyanate (The & Feltkamp, 1969) purchased from Mérieux (Lyon, France).

Results

Effect of preloading lysosomes with fluorescein-labelled anti-peroxidase

Quintart *et al.* (1979) showed by density equilibration studies that, after 1 h exposure of HTC cells to peroxidase, it is associated with particles of lower density than lysosomes. Upon further culture, the distribution of peroxidase becomes progressively similar to that of lysosomal enzymes. In cells pre-exposed to ribonuclease dimer, this modification of the distribution of peroxidase as a function of time does not occur and it is not degraded (Bartholeyns *et al.*, 1979).

To test the hypothesis that pretreatment of the cells with ribonuclease dimer could result in an inhibition of fusion between phagosomes and lysosomes, HTC cells were exposed to 0.1 mg of anti-peroxidase IgG/ml for 48 h. After washing, the cells were exposed to a medium containing ribonuclease dimer (0.05 mg/ml) and peroxidase (1 mg/

ml) for 2 h. Cells were further grown in the usual culture medium for 8 h. Control cells were treated the same way, but preloading with anti-peroxidase IgG was omitted. If the alteration of intracellular processing of peroxidase is due to an absence of fusion between phagosomes and lysosomes, previous accumulation of anti-peroxidase IgG should have no effect on the intracellular fate of peroxidase in ribonuclease-dimer-treated cells. The antibody may, however, force transfer of peroxidase into the lysosomes, if fusion is still taking place.

The subcellular localization of peroxidase was determined by fractionation of the homogenates by density equilibration in a sucrose gradient. The anti-peroxidase IgG was coupled with fluorescein, to check its association with lysosomes. Labelling of the antibody with fluorescein rather than isotopically was preferred, since studies on fibroblasts have shown that such labelling enhances uptake by the cells (Schneider *et al.*, 1979a). Fig. 1 shows that the specific antibody indeed displayed a lysosomal distribution. Comparison of cells preloaded with anti-peroxidase and of control cells shows that the antibody induced a shift of endocytosed peroxidase towards the higher-density region of the gradient. Furthermore, previous accumulation of anti-peroxidase IgG in lysosomes resulted in similar density distributions for peroxidase and for lysosomal *N*-acetyl- β -glucosaminidase. The density distributions for enzymes other than peroxidase were not significantly modified by preloading lysosomes with specific anti-peroxidase IgG. These results indicate that fusions between phagosomes and lysosomes are still occurring in the presence of the dimer, since previous accumulation of anti-peroxidase IgG restores the transfer of endocytosed peroxidase to lysosomes in dimer treated cells.

The effect of previous accumulation of anti-peroxidase IgG on the degradation of peroxidase taken up by the cells was studied. After endocytosis of peroxidase acetylated with [14 C]acetic anhydride, decay of radioactivity and of enzyme activity followed similar kinetics (Bartholeyns *et al.*, 1979). Furthermore, release of peroxidase in the culture medium here plays a minor role: it represents only 0.5% and 1.6% of the cell content for control and dimer-treated cells respectively. Since endogenous peroxidase activity can be neglected in our system, loss of enzyme activity essentially represents degradation of peroxidase taken up by the cells. The same conditions as for the experiments in Fig. 1 were used, except that the previous accumulation of anti-peroxidase IgG was separated from exposure to ribonuclease dimer and peroxidase by a period of 8 h culture in the usual medium. Furthermore, apart from cells not exposed to anti-peroxidase or to ribonuclease dimer, the controls included the replacement of anti-peroxidase by non-specific IgG

Fig. 1. Density equilibration of HTC cell homogenates after endocytosis of peroxidase in presence of ribonuclease dimer: effect of preloading with anti-peroxidase IgG

Ribonuclease dimer (0.05 mg/ml) was added to the culture medium together with peroxidase (1 mg/ml) for 2 h. Cells were further cultured for 8 h in the usual medium. Thick lines: exposure (48 h) of cells to 0.1 mg of anti-peroxidase IgG/ml before ribonuclease dimer. Thin lines: no preloading of cells with anti-peroxidase IgG. Recoveries ranged between 84 and 119% for all constituents. Distributions presented are the means of three experiments; areas corresponding to the sample layer and to the high-density cushion have been hatched. Particles in these regions are not necessarily at equilibrium. (a) Alkaline phosphodiesterase; (b) *N*-acetyl- β -glucosaminidase; (c) galactosyltransferase; (d) anti-peroxidase IgG; (e) dimerized ribonuclease; (f) peroxidase.

and omission of initial preloading with the antibody. Fig. 2 shows that previous accumulation of anti-peroxidase IgG resulted in a degradation of endocytosed peroxidase almost equivalent to that observed in cells not exposed to ribonuclease dimer, while previous accumulation of non-specific IgG was without effect.

Electron microscopy

HTC cells were first exposed to ferritin for 22 h to label lysosomes (Leroy-Houyet *et al.*, 1979), and then were exposed to dimerized ribonuclease (0.05 mg/ml) and allowed to endocytose peroxidase (1 mg/ml) for 8 h. In cells not exposed to ribonuclease dimer, the two markers were mainly

Fig. 2. Decay of peroxidase activity after endocytosis by HTC cells in the presence of ribonuclease dimer: effect of preloading with anti-peroxidase IgG

Results are expressed as % of initial peroxidase accumulations. ▲, 48 h uptake of anti-peroxidase IgG (0.1 mg/ml) followed by 8 h culture in fresh medium, and by 2 h uptake of peroxidase (1 mg/ml) and ribonuclease dimer (0.05 mg/ml) followed by further culture in fresh medium (uptake $0.95 \mu\text{g}$ of peroxidase/mg of cell protein). ▼, the same, but 48 h of uptake of non-specific IgG (0.1 mg/ml) before exposure to peroxidase and ribonuclease dimer. ●, 2 h uptake of peroxidase (1 mg/ml) and ribonuclease dimer (0.05 mg/ml) and further culture in fresh medium (uptake $0.87 \mu\text{g}$ of peroxidase/mg of cell protein). ■, 2 h uptake of peroxidase (1 mg/ml), washing and further culture (control cells; uptake $0.72 \mu\text{g}$ of peroxidase/mg of cell protein).

Fig. 3. Effect of galactose on degradation of peroxidase by dimer-treated cells

Cells were exposed to peroxidase (2 mg/ml) and ribonuclease dimer (0.05 mg/ml) for 2 h, then washed and further grown in fresh medium. Galactose (100 mM) was added to the medium at various times (arrows). The initial values for peroxidase accumulation were: ●, 1.20 (value chosen as 100%); ○, 1.25; ▲, 1.26 and ■, 1.22 μg /mg of cell protein.

observed in the same structures (Plate 1), although ferritin was sometimes masked by the intense peroxidase reaction. In dimer-treated cells (Plate 2), a number of large vesicles contained no ferritin, but peroxidase staining was observed at their periphery. Particles with the morphological characteristics of lysosomes, and labelled with ferritin only, were also present. Dissociation of the two markers was, however, incomplete; peroxidase-positive, ferritin-containing structures were also found. No other alteration was detected, except for some vacuolization of the cytoplasm.

Effect of galactose

Edelson & Cohn (1974a,b) have reported that the intracellular degradation of peroxidase by macrophages is inhibited by concanavalin A; this effect can be suppressed by subsequent addition of 100 mM-mannose to the medium (Edelson & Cohn, 1974b; Rosenberg & Charalampous, 1977). We investigated the action of sugars on dimer-treated hepatoma cells. Glucose, *N*-acetyl- β -glucosamine or mannose at 100 mM concentration did not have any effect on the degradation of endocytosed peroxidase

Fig. 4. Release of ^3H -labelled ribonuclease dimer into the culture medium induced by galactose

HTC cells were grown for 1 h in presence of ribonuclease A dimer (0.05 mg/ml) labelled with [^3H]acetic anhydride. The cells were then washed three times with fresh culture medium to remove free ribonuclease and further incubated in control medium (●) or in the presence of 100 mM-galactose (▲). Results are expressed as the amount of dimerized ribonuclease released in the medium, as a percentage of the total amount bound to the cells ($0.27 \mu\text{g}$ of dimer/mg of cell protein).

EXPLANATION OF PLATE 1

Electron micrograph of control HTC cells: sequential ferritin and peroxidase labelling

Cells were exposed to ferritin (5 mg/ml) for 22 h, washed and grown for 14 h in fresh medium and then exposed to peroxidase (1 mg/ml) for 8 h. After washing and fixation, the cells were stained cytochemically for peroxidase; the reaction products of peroxidase and ferritin occur mainly in the same structures. Magnification $\times 80\,000$.

EXPLANATION OF PLATE 2

Electron micrograph of dimer-treated HTC cells: sequential ferritin and peroxidase labelling

Cells were exposed for 8 h to dimerized ribonuclease and peroxidase after preloading with ferritin for 22 h as in Plate 1. Compared with control HTC cells, a higher number of vacuoles containing only the reaction product of peroxidase or only ferritin can be detected. The former often occurs within large structures which are rarely seen in control cells. Some vacuolization can also be observed in the cells exposed to dimerized ribonuclease. Magnification $\times 80000$.

by hepatoma cells exposed to ribonuclease dimer and thus were not studied further. Galactose, however, restored the intracellular digestion of peroxidase in ribonuclease dimer-treated cells. In the experiment shown in Fig. 3, HTC cells were exposed to ribonuclease dimer (0.05 mg/ml) and peroxidase (2 mg/ml) for 2 h, then washed, subdivided into four batches and further grown in fresh medium. Galactose (100 mM) was added at specific times. Even if galactose was added more than 5 h after exposure of the cells to dimerized ribonuclease, the degradation of endocytosed peroxidase started again.

If galactosyl residues on the plasma membrane are involved in the action of ribonuclease dimer, treatment of intact cells with neuraminidase could be expected to increase association of the dimer with the cells. Hence, HTC cells were pretreated at 37°C with 0.1 mg of neuraminidase/ml for 20 h, washed and exposed to 0.05 mg of dimerized ribonuclease/ml. We observed a 50% increase in the amount of dimer associated with the cells after 1 h of contact, compared with cells not exposed to neuraminidase.

The reversal by galactose of the effect of ribonuclease dimer was confirmed by two other experiments. Firstly, cells were grown for 1 h in the presence of dimerized ribonuclease (0.05 mg/ml) labelled with [³H]acetic anhydride. Cells were washed three times with fresh culture medium and

incubated in the usual medium, supplemented with 100 mM-galactose. Fig. 4 shows that addition of galactose induced the release of ribonuclease A dimer from the cells into the medium, although the release did not exceed 50% over a period of 2 h.

Secondly, HTC cells were grown for 4 h in the presence of ribonuclease dimer (0.05 mg/ml) and peroxidase (2 mg/ml). The cells were washed and cultured for 8 h in the usual medium supplemented with 100 mM-galactose. A homogenate was layered over a sucrose gradient and equilibrated by centrifugation. The distribution obtained is shown in Fig. 5; addition of galactose to the medium resulted in a distribution of previously endocytosed peroxidase comparable with that of lysosomal *N*-acetyl- β -glucosaminidase. This last enzyme displayed an unusually high peak at a density of 1.20, although its medium equilibrium density (1.197) was not significantly different from that observed in control experiments (1.200 ± 0.002 s.d., see Fig. 1). The distribution of ribonuclease A dimer in Fig. 5 was complex, but it also showed a peak concentration at a density of 1.20; the distribution was clearly

Fig. 5. Density equilibration of homogenates after endocytosis of peroxidase in the presence of ribonuclease dimer: effect of galactose

HTC cells were grown for 4 h in the presence of dimerized ribonuclease (0.05 mg/ml) and peroxidase (2 mg/ml). After washing, cells were grown for 8 h in medium containing 100 mM-galactose. Cells were homogenized and fractionated as described in the Materials and methods section; recoveries for the various markers were between 90% and 105%. (a) Alkaline phosphodiesterase I; (b) *N*-acetyl- β -glucosaminidase; (c) dimerized ribonuclease; (d) peroxidase.

Fig. 6. Decay of the activity of peroxidase taken up by HTC cells in the presence of lysozyme dimer: effect of the addition of galactose

■, control HTC cells grown for 2 h in the presence of peroxidase (2 mg/ml) before washing and re-suspension at time zero; ●, HTC cells grown for 2 h in presence of dimerized lysozyme (0.2 mg/ml) and peroxidase (2 mg/ml) before washing; ▲, HTC cells grown for 2 h in the presence of dimerized lysozyme and of peroxidase. After the uptake period, the cells were washed and grown in fresh culture medium to which 100 mM-galactose was added after 3 h of culture. Initial values for peroxidase accumulation were 1.2 and 1.5 μ g/mg of cell protein in the absence and presence of lysozyme respectively.

Fig. 7. Fixation of ribonuclease dimer by HTC cells in the presence of increasing concentrations of lysozyme dimer: competition for the fixation sites

HTC cells grown for 1 h in the presence of various concentrations of lysozyme dimer were exposed for 1 h to ribonuclease dimer (0.2 mg/ml) labelled with [^3H]acetic anhydride. Cells were then washed and the cell-associated radioactivity was measured.

different from that observed in the absence of galactose (Fig. 1).

Effect of dimerized lysozyme

A dimer of lysozyme, another basic protein, was prepared and its effect on intracellular peroxidase degradation was investigated. Lysozyme was chosen as it has been previously shown to inhibit proliferation of tumour cells in culture (Bartholeyns & Zenebergh, 1979). In the presence of dimerized lysozyme (0.2 mg/ml), the half-life of peroxidase increased from approx. 5 h to approx. 15 h (Fig. 6). Intracellular processing of the exogenous protein is thus also impaired by dimerized lysozyme, although the effect is not as pronounced as in the case of ribonuclease. Addition of 100 mM-galactose to the culture medium restored the intracellular degradation of peroxidase taken up in the presence of the lysozyme dimer. Ribonuclease and lysozyme dimers could therefore affect cells by a similar mechanism. Indeed, in experiments where cells were preincubated with various concentrations of lysozyme dimer, the latter clearly inhibited the subsequent fixation of labelled ribonuclease dimer by HTC cells exposed for 1 h to dimerized ribonuclease (Fig. 7).

Discussion

Interpretation of the present observations rests on the main characteristics of our previous experimental model (Bartholeyns *et al.*, 1979). Firstly, the

inhibition of the degradation of exogenous protein induced by dimerized ribonuclease is not due to an inhibition of lysosomal proteinases. Secondly, cell fractionation showed that endocytosed peroxidase is never associated with lysosomes in dimer-treated cells, whereas it is in control cells. Thirdly, ribonuclease dimer modifies the adsorptive properties of the pericellular membrane; fluid endocytosis accounts for the accumulation of peroxidase in control cells at the concentrations used (1–2 mg/ml in the culture medium), but adsorption to the pericellular membrane of cells exposed to the dimer increases the uptake 2.5-fold during the first 1 h.

The density equilibration analysis and study of the half-life of peroxidase in dimer-treated cells presented here show that pretreatment of the cells with fluorescent anti-peroxidase IgG corrects for the alteration of intracellular processing induced by ribonuclease dimer. Further, in the conditions used, the distribution of anti-peroxidase IgG observed after density equilibration of homogenates is similar to that of *N*-acetyl- β -glucosaminidase and clearly different from that of plasma membrane alkaline phosphodiesterase; this indicates association of anti-peroxidase IgG with lysosomes. Such localization is consistent with the observations of Schneider *et al.* (1979a,b, 1981) who have shown that fluorescent IgG is taken up rapidly by cultured fibroblasts and accumulates in lysosomes.

Two hypotheses have been proposed for the mechanism of action of ribonuclease dimer on hepatoma cells: an absence of fusion between phagosomes and lysosomes or an inhibition of the transfer of peroxidase adsorbed onto phagosomal membranes (Bartholeyns *et al.*, 1979). In our opinion, the experiments with specific anti-peroxidase IgG argue strongly in favour of the latter. The absence of effect of non-specific IgG upon peroxidase degradation in dimer-treated cells (Fig. 2) suggests a specific antigen-antibody reaction. The data in Fig. 1 also indicate a specificity of the effect of anti-peroxidase, since the distribution of dimerized ribonuclease does not show large modifications. Our observations cannot be explained if no contact occurs between peroxidase and its antibody in dimer-treated cells; therefore, it must be concluded that fusion between phagosomes and lysosomes still occurs in cells exposed to dimerized ribonuclease A. Since anti-peroxidase IgG is essentially localized in lysosomes, it is likely that ribonuclease dimer acts by impairing transfer of endocytosed peroxidase from phagosomes to lysosomes upon fusion of these organelles. From our data, a reaction between peroxidase and anti-peroxidase IgG at the plasma membrane cannot formally be excluded, but appears very unlikely since the fractionation data do not indicate the presence of antibody at the plasma membrane.

Our results can be explained if peroxidase fails to remain in the lysosomes of dimer-treated cells after fusion between lysosomes and phagosomes. The increased adsorption of peroxidase onto the plasma-membrane, shown previously in dimer-treated cells, is likely to result also in stronger binding of peroxidase on to the phagosomal membrane. This may allow peroxidase to escape digestion by lysosomal hydrolases in dimer-treated cells by remaining associated with phagosomal membranes with which it may eventually be carried back to the pericellular membrane by membrane-recycling (Heuser & Reese, 1973; Steinman *et al.*, 1976; Duncan & Pratten, 1977; Tulkens *et al.*, 1977; Silverstein *et al.*, 1977; Schneider *et al.*, 1979b). The affinity of anti-peroxidase accumulated in lysosomes for its antigen would, however, allow the desorption of peroxidase from the phagosomal membrane and its transfer to the lysosomal space for subsequent digestion. Schneider & Trouet (1981) have recently reported that chloroquine and methylamine inhibited the discharge in lysosomes of IgG endocytosed by fibroblasts and blocked the intralysosomal degradation of this IgG. They explained their data as a consequence of an impairment of dissociation of IgG from the membrane of endocytotic vesicles during transient fusion with lysosomes and also of the slowing down of plasma membrane recycling.

From our electron microscopic observations it can be inferred that, while some mixing of lysosomal and phagosomal contents is still occurring after exposure of the cells to the dimer, a large dissociation of the markers used to label lysosomes and phagosomes is observed. Peroxidase staining is also more restricted to the phagosomal-membrane area in dimer-treated cells.

Our observations are similar to those described by Edelson & Cohn (1974a,b) for concanavalin A-treated macrophages. These authors have interpreted their results as an inhibition of fusion between phagosomes and lysosomes, but they did not consider the possibility of inhibition of transfer, which cannot be excluded from their experimental data. The inhibition of fusion between lysosomes from macrophages and parasite-containing endocytotic vacuoles has been described (Armstrong & D'Arcy Hart, 1971; Jones & Hirsch, 1972; Jones *et al.*, 1972).

On a molecular basis, the mechanism by which dimerized ribonuclease binds to the cell membrane and promotes adsorption of peroxidase to the plasma membrane is not known. The effects of dimerized lysozyme on HTC cells confirm that the effect of dimerized ribonuclease is not related to its enzymic activity (Bartholeyns & Baudhuin, 1976). Furthermore, evidence has been presented for competition between lysozyme and ribonuclease dimers for the same membrane sites.

Addition of galactose to the cell culture medium rapidly abolishes the changes of intracellular protein processing induced by the dimers; peroxidase is transferred into the lysosomes and is degraded. The results shown in Figs. 4 and 5 suggest that galactose desorbs the dimer from the plasma membrane. The release of ribonuclease dimer into the medium is, however, incomplete and slower than expected from the rapid reversal of the effects of dimerized ribonuclease by galactose. After 2 h, a maximum 45% of the dimer bound to the cells could be released in the culture medium in the presence of galactose. This could be related to the heterogeneity of the dimer molecules caused by the fact that various lysine residues can react with the dimerizing agent. It should also be remembered that phagosomal membranes which are not accessible to the extracellular medium represent an area roughly equivalent to that of the plasma membrane in HTC cells (Leroy-Houyet *et al.*, 1979) and this may also explain incomplete release. As is shown by the fractionation data (Fig. 5), exposure of the cells to galactose results also in the transfer of dimerized ribonuclease to lysosomes.

The reversal of the effect of dimer by galactose confirms the importance of its fixation to the cell membrane. The desorption of the dimer is, however, rather surprising, since ribonuclease A is not a glycoprotein. On the basis of our results, the exact mechanism by which 100 mM-galactose influences the binding of dimerized ribonuclease to hepatoma cells cannot be defined.

Although the cross-linked dimer of lysozyme has similar effects to ribonuclease dimer on tumour cells, other basic proteins give relatively poor results; dimers of cytochrome *c* are not cytostatic and with histones or polylysines the effects cannot be observed due to their cytotoxicity (Bartholeyns & Zenebergh, 1979). Polylysine, for example, is fixed to the plasma membrane and induces cell lysis (Seljelid *et al.*, 1973; Mayhew *et al.*, 1974). Other polycationic substances bind to the cell membrane and act as multivalent ligands (Moroson, 1971; Seiler *et al.*, 1975; Larsen, 1977). These substances increase endocytosis of proteins by tumour cells (Shen & Ryser, 1978). Fixation of protein dimers on the cell membrane is probably related both to their basicity and to their dimeric structure; their effect on intracellular processing could probably be found also for dimers of other polycationic non-toxic proteins of low molecular weight that bind to the cells.

The effect of dimerized ribonuclease or lysozyme on HTC cells provides an experimental model which shows the importance of intracellular transfer for processing of molecules that penetrate the cell by adsorptive endocytosis. Fusion of phagosomes and lysosomes is not necessarily sufficient for pro-

cessing and must be accompanied by desorption of membrane-bound material. This last process may differ according to the type of molecule and may be influenced by other substances or by conditions such as the pH prevailing in the lysosomal space.

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