Inhibition of the Discharge of Endocytosed Protein from Phagosomes into Lysosomes in Hepatoma Cells Exposed to Dimerized Ribonuclease A

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The mechanism of the cytostatic action of dimerized ribonuclease A toward cultured hepatoma cells was investigated. A decrease in mitotic index, modifications of adsorptive properties of the pericellular membrane and inhibition of the degradation of two different proteins taken up by endocytosis are the first cell functions to be affected by the dimer. This effect on protein digestion is not due to an inhibition of proteolytic enzymes. The intracellular localization of exogenous protein and of ribonuclease dimer was studied by cell fractionation. When proteins (horseradish peroxidase or rabbit immunoglobulin G) are taken up by control hepatoma cells, they are first associated with phagosomes equilibrating at a lower density than lysosomes; their density distribution gradually becomes similar to that of lysosomes. When cells are pre-exposed to ribonuclease dimer, this modification of the density distribution as a function of time no longer occurs, although these proteins are still intracellular, as indicated by fractionation by differential centrifugation. During the first hour after addition of ribonuclease dimer, kinetic studies show an increased fixation of peroxidase to the cell membrane. Protein release into the culture medium is also increased. These results can be explained either by an absence of fusion between phagosomes and lysosomes, or by an inhibition of the discharge of peroxidase adsorbed to the phagosomal membrane after fusion.

A dimeric form of bovine pancreatic RNAase A can be obtained by cross-linking the enzyme to dimethyl suberimidate (Bartholeyns & Moore, 1974; Wang *et al.*, 1976). This synthetic dimer inhibits the proliferation of tumour cells when it is added to their culture medium. When injected in the vicinity of the tumour, it also decreases the development of tumours induced in rats with hepatoma cells (Bartholeyns & Zenebergh, 1978) or in mice with sarcoma ascites cells, and metastasis in lymph nodes and spleen of AKR/J leukaemic mice (Tarnowski *et al.*, 1977). These anti-tumoral properties of RNAase d confirm the observations made with the naturally occurring seminal-plasma RNAase dimer (D'Alessio *et al.*, 1972), which causes regression of sarcoma, hepatoma and Crocker tumours (Matousek, 1973; Stanek & Matousek, 1976). The cytostatic effect of RNAase d on different cell lines in culture was shown to be independent of its enzymic activity and to be largely a function of its rate of uptake by the cells (Bartholeyns & Baudhuin, 1976; Bartholeyns & Zenebergh,

1978). RNAase A, used at much higher concentration (Ledoux, 1956; Graffi & Arnold, 1973), or even some positively charged polymers (Katchalski *et al.*, 1964) also share this cytostatic effect.

The main purpose of the present work was to study the mechanism of action of RNAase d on tumoral HTC cells. Results showing that intracellular degradation of horseradish peroxidase taken up by endocytosis is one of the first cell functions to be affected after addition of 50 µg of dimer per ml of culture medium and not by RNAase A have been published in preliminary form (Bartholeyns & Baudhuin, 1977). This observation has been extended, and data are presented showing that RNAase d uptake by the cells results in impairment of the endocytic process.

Materials and Methods

Cell cultures

HTC cells, derived from Morris hepatoma 7288 C, were maintained 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: 9g of Swim-77 powder, 0.5g of NaHCO₃, 2g of glucose, 5mg of Phenol Red, 60mg of peni-

Abbreviations used: HTC cells, hepatoma tissue culture cells; IgG, non-specific rabbit immunoglobulin G; RNAase A, monomer of bovine pancreatic ribonuclease A; RNAase d, cross-linked dimer of bovine pancreatic ribonuclease A.

cillin G, 50nmol of Tris/hydroxymethylglycine and 5.5mg of cystine. After adjustment of pH to 7.85 the medium was supplemented with 10% (v/v) foetal calf serum. Cells (2×10^5 cells/ml initially) had a generation time of about 22h and grew exponentially up to 1.2×10^6 cells/ml.

Fractionation

Homogenates of HTC cells were prepared from 5×10^8 cells by suspension in 0.25M-sucrose and homogenization by 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 of Beaufay & Amar-Costesec (1976) in a Beckman L5-50 centrifuge (Beckman Instruments, Palo Alto, CA, U.S.A.). The whole homogenate (10ml) was layered on a 32ml linear sucrose gradient (density $1.08\text{--}1.27\text{ g/cm}^3$). To avoid formation of a pellet, a cushion of 6ml of sucrose solution of density 1.34 g/cm^3 was used. After centrifugation for 105min at 45000rev./min (time integral of squared angular velocity $W = 1.45 \times 10^{11}\text{ rad}^2 \cdot \text{s}^{-1}$), 13 or 14 fractions were collected. Their densities were measured as described by Beaufay *et al.* (1974b) and they were diluted in Triton X-100 (0.1 g/l) for analysis. Results are presented in the form of normalized histograms (de Duve, 1967).

Fractionation by differential sedimentation in 0.25M-sucrose was carried out as described by de Duve *et al.* (1955).

Assays

The methods used for the enzyme determinations can be found in the following references: neutral ribonuclease (EC 3.1.4.22) and acid deoxyribonuclease (EC 3.1.4.5), Bartholeyns *et al.* (1975); cytochrome oxidase (EC 1.9.3.1), Peeters-Joris *et al.* (1975); catalase (EC 1.11.1.6), Baudhuin *et al.* (1964); *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 galactosyl-transferase (EC 2.4.1.38), Beaufay *et al.* (1974a); phosphoglucomutase (EC 2.7.5.1), Ray & Roscelli (1964); DNA, Burton (1956); RNA, Fleck & Munro (1962); peroxidase (EC 1.11.1.7), Strauss (1958); acid mannosidase (EC 3.2.1.24), Libert *et al.* (1976); protein, Lowry *et al.* (1951).

Mitotic indices were determined after collection of the cells on slides in a Shandon Elliott Cyto-centrifuge and staining for 3min with 2% (w/v) Orcein in acetic acid.

The incorporation of [^3H]thymidine, [^3H]uridine and [^3H]leucine into trichloroacetic acid-insoluble material was performed as follows. Cell suspension (1ml) was incubated at 37°C in the presence of the labelled compounds (1 μCi , specific radioactivity

1Ci/mmol). After 1h the incubation mixture was diluted with 1mM-thymidine, -uridine or -leucine. The suspension was cooled to 0°C and centrifuged for 10min at 1000rev./min in an International centrifuge, model PR-J (International Equipment Co., Boston, MA, U.S.A.). After two washings, the pellet was resuspended in 1ml of culture medium, to which 4ml of 10% (w/v) trichloroacetic acid was added, and kept at 0°C. After 30min the sample was centrifuged for 20min at 2000rev./min and washed twice with 10% trichloroacetic acid. After resuspension in 1ml of 1M-NaOH, the preparation was left for 1h at room temperature, neutralized, and the radioactivity was measured.

Intracellular concentration of chloroquine or of fluorescent IgG was measured fluorimetrically, in a Perkin-Elmer 1000 fluorimeter (Perkin-Elmer Ltd., Beaconsfield, U.K.). The following wavelengths were used respectively for chloroquine and fluorescein: 340nm and 485nm for excitation, 370nm and 520nm for emission.

Release of horseradish peroxidase and RNAase d into the culture medium was studied in a serum-free medium in order to avoid interference of serum enzymes. For enzyme determinations, 5ml samples were taken from the culture medium at different times and concentrated approx. 50-fold on a Minicon sample concentrator (Amicon B.V., Oosterhout, The Netherlands).

Materials

Swim-77 powder for the preparation of culture medium and foetal calf serum were purchased from Gibco (Paisley, Scotland, U.K.). RNAase A (type I-A) and horseradish peroxidase (type II) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and dimethyl suberimide dihydrochloride was from Pierce Chemical (Rockford, IL, U.S.A.).

The cross-linked dimer of pancreatic RNAase A was prepared and labelled as previously described (Bartholeyns & Baudhuin, 1976). IgG was prepared by the method of Sober *et al.* (1956) and labelled with fluorescein isothiocyanate (The & Feltkamp, 1969) purchased from Mérieux (Lyon, France).

Results

Intracellular localization after uptake of horseradish peroxidase, IgG and RNAase d

Control cultures. The subcellular localization of peroxidase in control cultures was first studied by density equilibration of whole homogenates in a sucrose gradient. After exposure to peroxidase, the cells were either immediately homogenized or allowed to grow for 8h in the usual culture medium (Fig. 1). Immediately after its uptake, peroxidase is found mainly in the low-density region of the gradient, as reported previously (Quintart *et al.*,

Fig. 1. Density equilibration of homogenates after peroxidase uptake: control cultures

Thick lines: 1h uptake (peroxidase 5mg/ml), followed immediately by homogenization; thin lines: 2h uptake (peroxidase, 2mg/ml), followed by 8h culture in fresh medium before homogenization. The frequency is the relative amount of components/unit density increment, the total area of the histogram being equal to 1.0. For fractionation immediately after uptake, homogenization was performed with only 10 strokes of the Dounce homogenizer. Recoveries (sum of the content of all fractions with respect to the homogenate) range between 80 and 114% for all constituents. Surfaces corresponding to the top and the bottom of the gradient have been hatched, since these regions correspond to soluble proteins or to particles not necessarily at their equilibrium density.

1976). Its distribution is dissociated from that of lysosomal *N*-acetyl- β -glucosaminidase. A small peak of peroxidase activity is seen around a density of 1.25 g/cm³, but corresponds most probably to incompletely homogenized cells, since a peak in this region is also observed in the distributions of protein and cytochrome oxidase. However, when HTC cells are allowed to process further the peroxidase taken up over 8h, the distribution of peroxidase accumulated becomes similar to that of the lysosomal markers.

Localization of peroxidase in the cytosol of the intact cell being very unlikely, these results indicate that initially it is associated with phagosomes that are either of very low density or that are broken by the homogenization procedure; after a few hours, the protein taken up is found associated with the lysosomes.

Effect of RNAase d. When RNAase d and peroxidase were added simultaneously to the medium and maintained together for 2h, the distribution of peroxidase after 8h of further culture in normal medium was clearly different from that observed in control cultures. Peroxidase and RNAase d equilibrated in the low-density region (Fig. 2). For peroxidase, comparison with Fig. 1 shows that in the presence of RNAase d the distribution is similar to

that observed in control cultures immediately after the uptake of the enzyme. The equilibration of lysosomal markers is also modified by the addition of RNAase d to the culture medium: a bimodal distribution is observed for acid phosphatase and deoxyribonuclease, and a shoulder appears in the low-density region for acid mannosidase and *N*-acetyl- β -glucosaminidase. The median equilibration densities of those lysosomal enzymes are significantly different in control cultures and in the presence of RNAase d. Differences for other markers and protein are not significant, except for alkaline phosphodiesterase I.

The effect of RNAase d on the intracellular localization of another protein taken up from the culture medium, IgG, was also investigated; IgG was used here as a marker for the endocytic process and not for its immunological properties. Cells were exposed for 6h to a medium containing peroxidase (2mg/ml), IgG (0.2mg/ml) and RNAase d (50 μ g/ml); they were washed and further cultured for 8h in normal medium. Results of this experiment are presented in Fig. 3; the distributions of the three exogenous proteins accumulated are largely similar and are modified in the presence of RNAase d. The uptake of IgG was 1.2 μ g/mg of cell protein in control cultures and 2 μ g/mg of cell protein in the presence of

Fig. 2. *Density equilibration of homogenates after uptake of peroxidase, in the presence or absence of RNAase d*
Experimental procedures and presentation are as in Fig. 1. HTC cells were grown for 2h in a medium containing peroxidase (2mg/ml) and RNAase d (50 µg/ml). The cells were then washed and reincubated for 8h in normal culture medium (thick lines). Distributions observed under the same conditions but in the absence of RNAase are presented for comparison (thin lines). Recoveries range between 87 and 110% for all constituents; the distributions presented are means of three experiments, except for acid mannosidase (two experiments).

RNAase d. The distribution of IgG in control cultures is shifted to lower densities compared with that of peroxidase. As a result of the addition of RNAase d to the culture medium, the median equilibrium density of IgG is nevertheless lowered from 1.1838 to 1.1496 g/cm³. The distribution of RNAase d traced by radioactive labelling is similar to that of the enzyme activity; a shoulder in the distribution of RNAase activity in the higher-density region is the only significant difference, which may be due to the small amount of neutral RNAase usually present in HTC cells.

It appears thus from density-equilibration experiments that exposure of HTC cells to RNAase d induces modifications in the equilibrium density of

structures with which the exogenous proteins are associated.

Differential centrifugation in 0.25M-sucrose was performed to characterize the intracellular localization further (Fig. 4). The additional information provided by such a fractionation system is that a clear dissociation can be observed between the distributions of RNAase d and peroxidase on the one hand and the distributions of galactosyltransferase and alkaline phosphodiesterase I on the other hand; the latter are markers for the Golgi apparatus and the plasma membrane respectively.

Intracellular fate of peroxidase

Degradation of peroxidase was followed by

Fig. 3. Density equilibration of an homogenate after uptake of peroxidase and IgG, in the presence or absence of RNAse d

Experimental procedures and presentation are as in Fig. 1. HTC cells were grown for 6h in a medium containing [3 H]acetylated RNAse d (50 μ g/ml), peroxidase (2mg/ml) and fluorescent non-specific IgG (200 μ g/ml). The cells were washed and further incubated for 8h at 37°C in the usual culture medium before homogenization (thick lines). Distributions observed under the same conditions, except that RNAse d was not added, are presented for comparison (thin lines). Recoveries range between 76 and 110% for all constituents.

Fig. 4. Distribution patterns of constituents after fractionation of HTC cells by differential centrifugation

Thick lines: hepatoma cells were incubated for 2h in a medium containing peroxidase (2mg/ml) and RNAse d (50 μ g/ml), and then grown for 8h in culture after resuspension in the usual medium. Thin lines: same experiment, except that RNAse d was omitted. The ordinate shows the relative specific activity of the fractions (% of total activity/% of total protein), and the abscissa represents the relative protein content, cumulatively in the order in which fractions were isolated. N, nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, supernatant. The relative protein content used for the calculation of results is the mean of two experiments. Recoveries range between 86 and 109% for all constituents.

Table 1. *Effect of RNAase d on the decay of peroxidase activity in HTC cells*

After pretreatment, cells were washed 3 times with fresh culture medium and further cultured; peroxidase activity was then followed on 5 ml portions removed at different times. For the enzyme determination, cells were washed 3 times with NaCl (9 g/l) and lysed by sonication in 1 ml of Triton X-100 (1 g/l). The results are expressed as means $\pm$ s.d.

Number of experiments	Pretreatment	Peroxidase accumulated after pretreatment (μ g/mg of cell protein)	Half-life of peroxidase activity (h)
6	Peroxidase (1 mg/ml), 4h	0.77 ± 0.06	5 ± 0.7
4	Peroxidase (1 mg/ml) and RNAase d (50 μ g/ml), 4h, simultaneously	0.84 ± 0.06	>40 in all cases
3	Peroxidase (1 mg/ml) and RNAase d (50 μ g/ml), 1h, simultaneously	0.49 ± 0.03	30 ± 6
2	Peroxidase (1 mg/ml), 4h, and RNAase d (50 μ g/ml), 1h, sequentially	0.72 ± 0.05	6 ± 0.6

Fig. 5. *Effect of RNAase d on the decay of peroxidase activity after endocytosis*

Results are expressed as a percentage of the initial activity/mg of cell protein. Δ , Control; $\blacktriangle$, simultaneous treatment with peroxidase and RNAase d over 4h; $\circ$, simultaneous treatment with peroxidase and RNAase d over 1h; $\bullet$, sequential treatment with peroxidase (4h) and then RNAase d (1h). The vertical bars refer to standard deviations. Details of the experimental conditions are given in Table 1.

measuring the enzyme activity after preloading the cells with it, in the presence or absence of RNAase d. The results of these experiments are illustrated in Fig. 5, and a detailed description of the pretreatments can be found in Table 1, together with evaluations of intracellular uptake and of the half-life of enzyme activity in the various conditions. The decay of peroxidase activity follows first-order kinetics over the

sampling period. The kinetics were similar in experiments where the decay of peroxidase radioactivity (acetylated with [14 C]acetic anhydride) was followed instead of the enzyme activity. The loss of enzyme activity, reflecting the degradation of the protein, occurs more than 5 times more slowly in cells exposed to RNAase d either before or during the preloading of cells with peroxidase (Fig. 5). Most of the RNAase d action is exerted within 1h of contact with the cells. However, exposure of cells to RNAase d has negligible effect on the intracellular degradation of peroxidase if RNAase d is added to the culture medium after peroxidase uptake. Although the action of the RNAase d appears to be relatively fast, it does not affect the degradation of peroxidase that is already within the cells.

We have also verified that the release of peroxidase into the culture medium plays only a minor role in the decay of intracellular peroxidase. In control cultures, this release is linear over a period of 5h and it represents per h on average ($\pm$ s.d.) $0.50\% (\pm 0.15\%)$ of the enzyme accumulated at the end of the uptake period. In cells exposed simultaneously to RNAase d and peroxidase over 4h, this release amounts to $1.6\% (\pm 0.5\%)$ per h. The release of acid hydrolases into the culture medium of the cells is also increased in the presence of RNAase d; for *N*-acetyl- β -glucosaminidase, this release amounts to 0.13% per h in control cells and 0.62% in the presence of RNAase d.

Kinetics of peroxidase uptake

Two phases can be distinguished in the kinetics curve of peroxidase uptake (Fig. 6): at the beginning, peroxidase is fixed in significantly ($P < 0.01$ at 2h) larger amounts by cells exposed to RNAase d. During the second phase, which starts from 3h onwards, accumulation of peroxidase is no longer significantly different in dimer-treated and control cultures. The difference observed during the first hour can largely be attributed to adsorption of peroxidase or a peroxidase-RNAase d complex to the plasma

Fig. 6. Uptake of peroxidase by HTC cells

HTC cells were cultured at 37°C in a medium containing 2mg of peroxidase/ml and in the absence (●) or presence (▲) of RNAase d (50 µg/ml). Samples were removed at different times, and the cells were washed 3 times with culture medium and 2 times with NaCl (9g/l). Preparations were then homogenized by sonication in Triton X-100 (1g/l) and tested for peroxidase activity and protein content.

Fig. 7. Intracellular fate of RNAase d

Cells were incubated in the presence of [³H]acetylated RNAase d (50 µg/ml). Experimental procedures are as in Fig. 6. The preparations were tested for neutral ribonuclease activity (●), and radioactivity (▲); the results presented are means ± s.d. for four experiments.

membrane. More surprising is the fact that during the second phase the accumulation of peroxidase is roughly the same, whether the cells have been exposed to RNAase d or not. Indeed, the inhibition of peroxidase degradation shown previously should result in greater accumulation of the protein in RNAase d-treated cells. The small increase in exocytosis of peroxidase after treatment does not compensate for the lack of its degradation. A direct effect of the dimer on endocytosis of peroxidase must thus be inferred. Indeed, if the cells exposed to RNAase d are washed and cultured for 8h in the usual culture medium before uptake of peroxidase over 4h, the intracellular peroxidase activity is only 60% of the value found for cells not exposed to RNAase d.

Intracellular fate of RNAase d

The activity of RNAase d taken up by the hepatoma cells decreases rather rapidly, with a half-life of about 3h (Fig. 7). However, when RNAase d was traced by ³H-labelling, only 10% of the label was lost over a period of 8h. The amount released in the medium is approximately the same as for peroxidase measured in the same conditions: 1.3%/h of the RNAase d accumulated by the cells (s.d.: 0.2%). RNAase d activity appears thus to be somehow masked inside the cells; as shown in Fig. 3, the intracellular localization of radioactively labelled RNAase

d or of the RNAase activity is, however, very similar. Treatment of cell homogenates with 0.25M-H₂SO₄, a usual procedure for purification of the enzyme, restored 90% of the activity.

Effect of RNAase d on other cellular functions

The other cellular functions studied (Fig. 8), except for the mitotic index, are only affected after endocytosis and digestion. Cell viability, measured by Trypan Blue exclusion, decreases only 20h after addition of RNAase d; about 25% dead cells are observed after 24h; most of our experiments were performed between 4 and 8h after addition of RNAase d, and no cytotoxicity could then be observed.

We have also tested if the ability of lysosomes to accumulate weak bases, in the present case chloroquine (Wibo & Poole, 1974), was affected by treatment of the cells with RNAase d. On the basis of experiments on fibroblasts, de Duve *et al.* (1974) have interpreted the accumulation of weak bases in the lysosomes of cells as being largely dependent on the acid pH prevailing in these organelles. Immediately after exposure to RNAase d, the accumulation of chloroquine by HTC cells was at first not significantly different from accumulation by control cells; however, a 40% decrease in the amount of chloroquine accumulated was observed after 16h, and a

Fig. 8. Effect of RNAase d on various cell functions. Cells were incubated at 37°C in the presence of RNAase d (50 µg/ml) and then of [³H]thymidine (▲), [³H]uridine (□) or [³H]leucine (■) as described in Materials and Methods section; mitotic indices (●) were also determined.

nearly 10-fold decrease was observed after 18 h exposure.

The effect on protein degradation and the modifications of the adsorptive properties of the plasma membrane, together with the decrease in the mitotic index, are thus the earliest events observed after exposure of HTC cells to RNAase d.

Discussion

Effect of RNAase d on the uptake of peroxidase

In view of the adsorption phenomena observed for peroxidase in cells exposed to RNAase d, a critical evaluation of the amount of exogenous protein taken up by HTC cells either as a solute or bound to membranes is necessary. Following the terminology used by several workers (for reviews, see de Duve *et al.*, 1974; Silverstein *et al.*, 1977), these two mechanisms for intracellular penetration will be termed fluid and adsorptive endocytosis respectively.

In control experiments, for cells exposed for 1 h to peroxidase the density distribution observed for peroxidase could reflect an association with phagosomes or with the plasma membrane; further, we have shown that peroxidase is transferred into the lysosomes within a few hours. On the basis of measure-

ments of average cell volume (2700 µm³) with a cell counter, and protein determinations, the HTC cells used in this work contain on average 0.4 ng of protein per cell. The volume fraction of the structures labelled by exposure of the cells to peroxidase for 1 h has been estimated in this laboratory to be 4.4% (M.-A. Leroy-Houyet, personal communication). Since after exposure for 1 h at 37°C to peroxidase (2 mg/ml) control culture cells contain 0.38 µg of peroxidase/mg of cell protein, the concentration of peroxidase within phagosomes is 1.3 mg/ml. There is thus no need to resort to adsorptive endocytosis to explain the rate of peroxidase uptake; our results are compatible with peroxidase uptake occurring by fluid endocytosis in control cultures, as concluded for macrophages by Steinman & Cohn (1972).

After exposure to 0.05 mg of RNAase d/ml and 2 mg of peroxidase/ml for 1 h at 37°C, the cells contain 0.9 µg of peroxidase/mg of cell protein (Fig. 6). The 2.5-fold increase in uptake may be considered as being largely due to adsorption to the cell membrane. At higher concentration of RNAase d (1 mg/ml) an even larger stimulation of uptake is obtained.

Effect of RNAase d on the subcellular localization of endocytosed protein

Density-equilibration analysis was performed on whole homogenates of HTC cells in this work. The resolution obtained compares favourably with that of similar experiments on postnuclear supernatants (Lopez-Saura *et al.*, 1972), and it has the advantage that populations of subcellular components are analysed in their totality. In control cultures, the density distribution of peroxidase 1 h after its uptake is clearly different from that of lysosomal hydrolases. An association of a large part of peroxidase with the plasma membrane can be ruled out by the dissociation between peroxidase and alkaline phosphodiesterase I in differential-centrifugation experiments. It should, however, be stressed that peroxidase could have been desorbed by washing the cells with whole culture medium and NaCl after the uptake period, as was routinely the case. We may thus conclude that within the first hour of its uptake peroxidase is still largely associated with phagosomes in control cultures.

The distribution of RNAase d in differential-centrifugation experiments (Fig. 4) is in agreement with previously reported results (Bartholeyns & Baudhuin, 1976), from which accumulation of RNAase d in lysosomes was inferred. The more detailed data from density-equilibration experiments presented here show, however, that structures containing RNAase d, which are concentrated in the L fraction, can be, at least partially, resolved from lysosomes, on the basis of the density distribution.

In control cultures, transfer of peroxidase and IgG from phagosomes to lysosomes is shown by the

increase in equilibrium density of the structures with which these proteins are associated and the similarity observed after 8 h for the distributions of exogenous proteins and lysosomal hydrolases. This modification of density distributions as a function of time is no longer observed after treatment of the cells with RNAase d. These exogenous proteins and RNAase d have entered the cell, since they are associated with an L fraction. Moreover, density distributions of lysosomal hydrolases are also slightly modified by RNAase d; the median equilibrium densities of acid hydrolases are displaced toward lower density.

Mechanism of action of RNAase d on the intracellular processing of exogenous protein

Inhibition of lysosomal proteinases can be ruled out as an explanation of the inhibition of peroxidase degradation, since peroxidase taken up by the cells before exposure to RNAase d is digested at a nearly normal rate. Moreover, the normal accumulation of chloroquine over the first few hours after addition of RNAase d can be interpreted as indicating that the acid pH of lysosomes is maintained. The observations on intracellular digestion reported here are analogous to those reported by others for concanavalin A (Goldstein *et al.*, 1975; Edelson & Cohn, 1974*a,b*), which inhibits the degradation of lipoproteins in human fibroblasts and of peroxidase and albumin in mouse macrophages. In the last case, the increase in the half-life of the protein taken up was 2.1- and 2.6-fold, whereas we observed a 9-fold increase in our experiments.

A modification of the properties of the plasma membrane may occur after addition of RNAase d. Most of the effect of RNAase d takes about 1 h, and it results in an impairment of the transfer of endocytosed protein to lysosomes. The present data are compatible with two possible explanations for this effect.

One hypothesis is that there is a marked inhibition of fusion between phagosomes and lysosomes. In their study on the effect of concanavalin A on macrophages, Edelson & Cohn (1974*b*) interpreted their morphological data as being a consequence of an inhibition of fusion. An absence of fusion has also been described between lysosomes and phagocytic vacuoles containing living parasites in macrophages (Armstrong & D'Arcy Hart, 1971; Jones & Hirsch, 1972; Goren *et al.*, 1976). In our study, fractionation data show that the inhibition was not complete; some limited fusions must have occurred over a period of 8 h, in order to explain the modification of the distribution of lysosomal hydrolases in RNAase d-treated cells.

A second possibility is inhibition of the unloading of peroxidase from the endocytic vacuoles into the lysosomes. The desorption of peroxidase adsorbed on the phagosomal membrane could be impaired

after fusion between phagosomes and lysosomes. In that case, in view of the membrane flow and recycling observed during endocytosis (Schneider *et al.*, 1977; Silverstein *et al.*, 1977; Steinman *et al.*, 1976), absorbed peroxidase would leave lysosomes with the membrane components returning to the cell periphery. The displacement of lysosomal hydrolases observed in the presence of RNAase d may then correspond to the population of phagolysosomes existing at the time of homogenization. This second interpretation could also account for the increase in secretion of lysosomal hydrolases; it is indeed possible that, in the presence of RNAase d, recycling of phagosomal membrane coated with RNAase d and peroxidase results in a somewhat higher transfer of lysosomal enzymes to the plasma membrane.

The direct consequences of the impairment of lysosomal digestion on cell functions and multiplication cannot be assessed at the present time. The correlation obtained between the rate of intracellular uptake and the inhibition of cell multiplication, together with the intracellular localization of RNAase d, suggests that the phagolysosomal system is involved in the cytostatic effect; however, modifications in the properties of the plasma membrane could be the primary cause that blocks both cell multiplication and transfer from phagosomes to lysosomes.

The cytostatic properties of RNAase d, and to some extent its mechanism of action, could be shared with some other positively charged polymers binding to a negatively charged membrane (see Katchalski *et al.*, 1964; Seljelid *et al.*, 1973); these questions together with the problems of cell specificity have been presented (Bartholeyns & Zenebergh, 1978). Although no systematic survey was performed, it should be pointed out that, up to now, the effect of RNAase d has only been found on tumoral cells; endocytosis of RNAase d is low in fibroblasts and macrophages, for which no effect on cell multiplication can be observed.

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