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EFFECT OF EMETINE AND CHLOROQUINE ON PHAGOCYTIC PROCESSES OF RAT MACROPHAGES

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Abstract—Emetine and chloroquine caused a dose-dependent and time-dependent inhibition of bacterial phagocytosis in rat peritoneal macrophages. The effect of emetine was found to be stronger: 50% inhibition was achieved at 5×10^{-6} M concentration. The inhibition of phagocytosis was irreversible. Neither the binding of bacteria to the surface of phagocytes nor membrane marker 5′ nucleotidase was touched by this treatment. The adherence of macrophages to plastic dishes was also impaired by the drugs but only in a smaller extent.

Both emetine and chloroquine blocked the amino acid incorporation into macrophage proteins. The effect of emetine was more marked; 10^{-6} M decreased the incorporation to 25% of control, while 10^{-4} M chloroquine produced a similar inhibition. The block of protein synthesis was also irreversible.

It is well established that mononuclear phagocytic cells have important functions both in afferent and efferent stages of the immune response. Macrophages play an important role in the elimination of various particles by endocytosis [1]. Furthermore they produce several enzymes [1, 2] and other factors [3] which are very likely responsible for immune modulation. The production of these biologically active factors depends on the culture conditions [4].

Recently the role of macrophages in antigen presentation has been investigated widely. Lysosomotropic amines like chloroquine and ammonium ions were utilized as tools for the study of this process [5–7]. An important effect of lysosomotropic drugs was observed in the inhibition of protein catabolism due to their increasing effect on lysosomal pH [7]. Allen and Unanue [8] reported different requirements for the processing of native and denatured proteins or their degraded sequences. Ammonium ions were found to inhibit the functions of phagosomes and lysosomes [9].

Chloroquine contains a quinoline ring in its structure; another alkaloide, emetine consists of two isoquinoline rings. The inhibitory effect of emetine on macromolecular biosynthesis *in vitro* in human tonsillar lymphocytes was published earlier [10]. Emetine also exerts influence on specific antibody production *in vivo* as reported recently by us [11].

In this report we intend to characterize and to compare the different inhibitory effects of emetine and chloroquine on rat peritoneal macrophages. Bacterial phagocytosis and binding, macrophage adherence, 5′ nucleotidase and protein synthesis were studied. It is interesting to note that the use of chloroquine and emetine as antiparasitic drugs makes an additional importance for these observations.

MATERIALS AND METHODS

Macrophages. CFY inbred rats (LATI, Gödöllö) were treated i.p. with 5.0–5.0 ml thioglycollate broth. The animals were sacrificed on the fourth day, and the macrophages were removed from the peritoneal cavity by using Ca^{2+}–Mg^{2+}-free Hanks medium. The exudate was centrifuged at 600 *g* for 10 min and a cell suspension ($1–2 \times 10^6$ cells per ml) was prepared in Hanks medium containing Ca^{2+} and Mg^{2+}. 2.0–2.0 ml of this suspension were left to adhere in plastic petri dishes (Nunc, Denmark) for 30 min at 37°. Non-adherent cells were removed by washing the adherent population twice with PBS (pH = 7.4).

Determination of bacterial phagocytosis. The method of Vray *et al.* [12] was applied. Briefly, *M. luteus* bacteria were labeled with FITC, labeled bacteria were opsonized with rat serum (dil. 1:5 as a complement source) for 15 min at 37°. Macrophages were incubated under various conditions (see Results) before adding opsonized bacteria (5×10^8 bacteria in 100 μl PBS). Ingestion was stopped by adding NEM (0.125 mg per ml final conc.), cells were washed twice with PBS (pH = 7.0) the non ingested bacteria were removed by lysozyme treatment. Finally, macrophages were solubilized in 1.0 ml 1% NP-40 detergent and the solubilized fluorescence was monitored in a Hitachi spectrofluorimeter. The phagocytic index was calculated and given in ingested bacteria per macrophage per hour.

Determination of binding of bacteria to macrophages. The binding was determined in a similar way as the phagocytosis but the cells and bacteria were incubated for 60 min at 4° in the presence of NEM. Lysozyme treatment was omitted and the unbound

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Abbreviations used: FITC, fluoresceine iso-thiocyanate; NEM, *N*-ethylmaleimide; NP-40, a non-ionic produced by BDH; PBS, phosphate buffered saline; PCA, perchloric acid.

bacteria were removed by excessive washing with PBS (pH = 7.0).

Treatment with emetine or chloroquine. Adherent macrophages were incubated in Eagle medium containing 10% autologous serum (decomplemented) and the drug. In the experiments when the effect of pre-incubation was studied the drug was removed before ingestion by washing twice with PBS; in other experiments the drug was added again into the fresh medium after washing.

The effect of the drugs on adherence. The effect was studied in two ways: (i) when the real adherence was investigated the drug was applied to the Ca^{2+}–Mg^{2+}-free Hanks medium; (ii) when the effect on the adhered cells was studied, the drugs were added to the Eagle medium. The adhered cells were labeled with ^{51}Cr [13], the labeled cells were solubilized in 1.0 ml 1% NP-40 and radioactivity was measured in a gamma-scintillation spectrometer (Gamma Works, Hungary).

5′ Nucleotidase assay. 2×10^6 adhered cells were washed three times with 0.15 M NaCl and cells were disrupted by freezing and thawing. The enzyme was measured according to Heppel and Hilmoe [14] on the basis of P_i release. Inorganic phosphate was determined by the method of Chen *et al.* [15].

^{14}C-labeled leucine incorporation. 3×10^6 macrophages were adhered. The adhered cells were pre-treated in Eagle medium containing 10% rat serum and the drug (and in Eagle medium only as a control) for 60 min at 37°. The medium was removed from the cells by washing them twice with PBS (pH = 7.4). Afterwards 1.0 ml Hanks medium was added. 74 kBq ^{14}C-leucine (sp. act. 469.5 MBq/mmol) was added to the cells for 60 min at 37°. After the incubation 1.0 ml 1 N PCA was added, the precipitate was transferred into centrifuge tubes and pelleted at 3000 *g* for 5 min. The precipitate was washed threefold with 1.0–1.0 ml 0.5 N PCA. Finally it was dissolved in 0.5 ml 0.5 N NaOH and radioactivity and protein were determined [16]. Incorporation was calculated in ^{14}C cpm per μg protein.

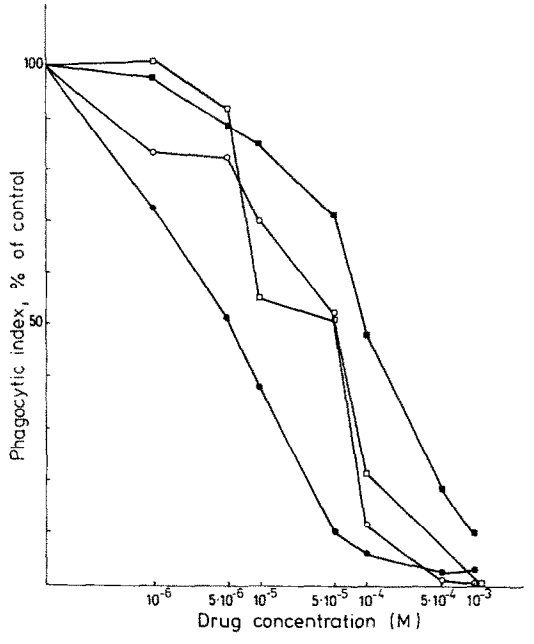

Fig. 1. The effect of drugs at various concentrations on the C3b-mediated bacterial phagocytosis of rat peritoneal macrophages. Macrophages were elicited by thioglycollate broth in vivo. Symbols: pre-treatment for 60 min with emetine (●), treatment with emetine for 60 min only during ingestion without pre-treatment (■), pre-treatment for 60 min with chloroquine (○), treatment with chloroquine for 60 min during ingestion only, without pre-treatment (□). Control: 8.68±2.25 phagocytized bacteria per macrophage per hour (considered as 100%). Samples without pre-treatment were incubated in Eagle medium containing 10% rat serum for 60 min. pre-treatment period.

RESULTS

1. The effect of emetine and chloroquine on bacterial phagocytosis was studied under various circumstances as shown in Fig. 1. Macrophages were (a) pre-incubated for 60 min in the presence of the drugs and after washing, the ingestion was performed in drug-free Eagle-medium (indicated briefly as "pre" samples), (b) pre-incubated for 60 min in the absence of the drugs, but the ingestion was performed in a medium containing the drug for 60 min (indicated as "same" samples). Emetine had a more marked effect in "pre" samples but in "same" samples the effect of chloroquine was slightly stronger. 50% inhibition was reached in "pre" samples at 5×10^{-6} M for emetine and at 5×10^{-5} M for chloroquine, while in "same" samples this was achieved at 10^{-4} M for emetine and at 2×10^{-5} M for chloroquine (Fig. 1) respectively.

2. For testing the time-dependence of the drug treatment an end concentration of 5×10^{-5} M was chosen (Fig. 2). In this case "pre" samples were incubated for the periods shown in the presence of the drug and after washing, the ingestion was performed in a drug-free medium for 60 min. In "same" samples a 60 min preincubation was done in the absence of the drug (in Eagle medium) and after changing the media the samples containing the drugs were incubated in Eagle medium for the period shown in Fig. 2 for the ingestion. In the case of the "pre" samples, a more marked effect was observed with emetine (50% inhibition was reached at 8.5 min) than with chloroquine (50% inhibition at 22.5 min), while in "same" samples chloroquine had a slightly stronger influence (50% inhibition at 42 min for emetine and 36 min for chloroquine).

3. The reversibility of the treatment was also studied. Macrophages were treated for 60 min in the presence of 5×10^{-5} M drug, then the drug was removed, cells were washed and transferred into a fresh Eagle medium containing 10% rat serum. Ingestion was monitored immediately in serum-free Eagle medium at 30, 60 and 120 min after removal of drugs. Table 1 shows that phagocytosis was not restored even 120 min after removing the drug, therefore the effects at least at this concentration may be considered irreversible.

4. The binding of complement-opsonized *M. luteus* bacteria to the cell surface could not be inhibited by the drug treatment if pre-incubation were applied (Table 2). The plasma membrane bound marker

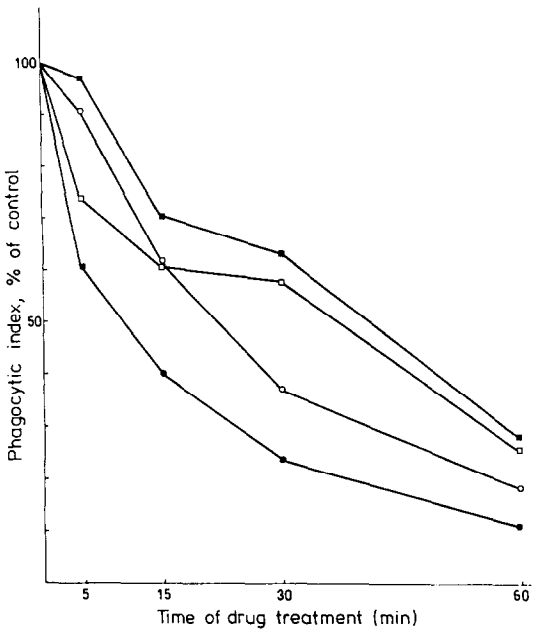

Fig. 2. Effect of drugs on the C3b-mediated bacterial phagocytosis of rat peritoneal macrophages. Dependence on treating periods: macrophages were elicited by thioglycollate broth *in vivo*. Symbols: pre-treatment with emetine (●), treatment with emetine during the ingestion without pretreatment (■), pre-treatment with chloroquine (○), treatment with chloroquine during the ingestion without pre-treatment (□). "Pre": Pre-treatment was carried out in Eagle-medium containing 10% serum for 60 min but drug was added only for the indicated period. After pre-treatment samples were washed by PBS, and phagocytosis was studied in drug free medium. "Same": Other samples were incubated in Eagle medium containing 10% serum for 60 min; after washing with PBS ingestion was carried out for 60 min and drugs were applied only for the indicated periods. Control: 6.882±1.94 phagocytized bacteria per macrophage per hour (considered as 100%).

enzyme, 5′ nucleotidase was not affected by the drugs (Table 3).

5. The effect of drug treatment on the adherence of peritoneal macrophages was also investigated. Lower concentrations had no effect. The highest applied dose (1 mM), however, caused a total arrest in the bacterial phagocytosis while decreasing the adherence by only 50%. Similar results were obtained when previously adhered macrophages were treated in the presence of the drugs for 60 min (Fig. 3).

Table 2. Effect on emetine and chloroquine on the binding of bacteria to macrophage surface

Concentration of drugs (M)	Binding index at 4° Emetine	Chloroquine
Control	9.31 ± 1.01	4.88 ± 0.07
10^{-6}	9.43 ± 0.43	5.92 ± 1.12
5×10^{-6}	9.46 ± 1.15	N.D.
10^{-5}	10.15 ± 0.46	6.90 ± 4.50
5×10^{-5}	7.65 ± 0.57	4.50 ± 1.05
10^{-4}	10.04 ± 0.86	3.53 ± 1.57
5×10^{-4}	7.45 ± 3.26	5.62 ± 0.98
10^{-3}	6.30 ± 0.09	5.77 ± 1.13

Binding index is given in attached bacteria per macrophage per hour; S.E.M. values were calculated from three simultaneous experiments.

6. Both emetine and chloroquine caused a marked decrease in the ^{14}C-leucine incorporation in a dose-dependent manner as shown in Fig. 4. The effect of emetine was more marked: even at 10^{-6} M dose it decreased the ^{14}C-leucine incorporation to 25% of the control and differences between "pre" and "same" samples were practically not observed. Similar results were observed at about 10^{-4} M when chloroquine was tested in "pre" samples. This compound had only moderate effects in "same" samples (Fig. 4), however.

7. The time dependence of the amino acid incorporation is shown in Fig. 5. The marked effect of emetine can also be seen in this respect, in 5 min the incorporation was practically stopped in "pre" samples, while "same" samples and chloroquine-treated samples the inhibition was found to be less than in emetine-treated "pre" samples.

8. The reversibility of the treatment was studied as mentioned in [3]. Similarly to the ingestion, the inhibition of the amino acid incorporation was also irreversible even after 120 min restoration (Table 4).

DISCUSSSION

Macrophages play an important role in the immune response by means of their phagocytic capacity, among others. The phagocytic process takes place in three stages, (1) the attachment (or

Table 1. The irreversible effect of emetine and chloroquine on phagocytic index

Time after removing of the drug (min)	Phagocytic index with emetine (%)	Phagocytic index with chloroquine (%)
Control	100	100
0	9.67	26.07
30	7.63	10.54
60	4.07	8.33
120	1.53	9.68

Treatment with the drugs was performed for 60 min at 5×10^{-5} M. Control: 9.82 ± 1.07 and 7.44 ± 0.87 ingested bacteria per macrophage per hour for emetine and chloroquine, respectively.

Table 3. Effect of emetine and chloroquine on the 5′ nucleotidase activity in macrophages

Concentration of drugs (M)	5′ nucleotidase spec. activity Emetine	Chloroquine
Control	6.65 ± 0.22	6.98
10^{-6}	6.57 ± 0.65	6.56
5×10^{-6}	6.94 ± 0.33	N.D.
10^{-5}	6.39 ± 0.64	7.26
5×10^{-5}	6.93 ± 0.67	6.66
10^{-4}	6.75 ± 0.75	7.75
5×10^{-4}	5.97 ± 1.10	7.01
10^{-3}	6.44 ± 0.76	8.21

5′ nucleotidase specific activity is given in fmole liberated P_i per macrophage per minute. S.E.M. values were calculated from three simultaneous experiments, the average of two experiments is given for chloroquine treatment. N.D. = not determined.

binding) of the particle to specific surface receptors; (2) the signal transmission from the receptors to the contractile systems involved in the internalization; (3) the ingestion itself. Finally, the phagocytized particles are subjected to a degradation process in the phagolysosomes.

The recognition and attachment of the particle is generally mediated by the membraneous Fc and C3b receptors [17, 18]. The rate of phagocytosis can be enhanced by opsonization with IgG and/or complement due to the increase of binding to the surface receptors. No damaging effect on these surface-associated functions was observed with any of the drugs (Table 2). The 5′ nucleotidase activity was also unchanged. These observations suggested that the inhibition of phagocytosis by the drugs was due neither to their effect on the macrophage surface nor to the attachment of particles.

The energy requirement of ingestion has been known for a long time [17, 19], and may be explained by the participation of myosin or actin-like contractile proteins in this process. The energy supply of phagocytosis is provided by glycolysis. If this process were blocked, the inhibitory effect should be reversible [17, 20] when drugs are removed, although the irreversible effects suggested that these compounds could not be washed out completely. Nevertheless, protein synthesis inhibitors are not known as blocking agents on glycolysis, so it is not likely that the inhibition may be attributed to the blocking of glycolysis.

Both emetine and chloroquine caused a reduction in the adherence of macrophages to the petri dishes when adherence was monitored by 51-chromium labeled macrophages. The adherence is considered

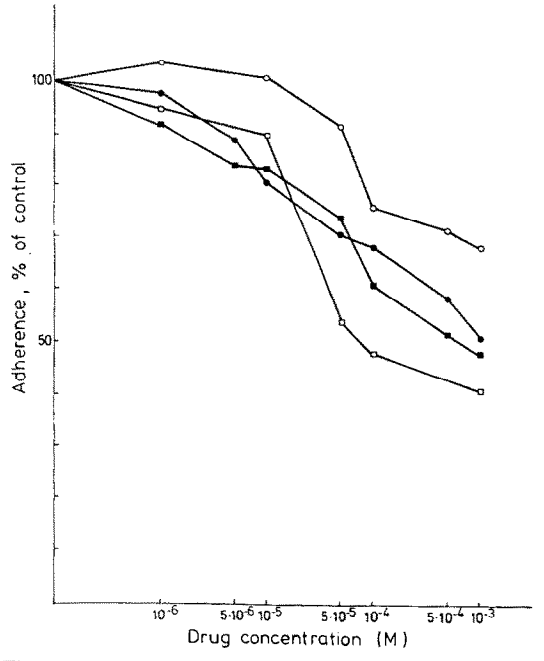

Fig. 3. Effect of drugs on adherence of rat peritoneal macrophages elicited by thioglycollate broth *in vivo*. Symbols: effect of emetine on the adherence of freshly isolated peritoneal macrophages (●), effect of emetine on ceasing of adherence of previously adhered cells (■), effect of chloroquine on the adherence of freshly isolated peritoneal macrophages (○), effect of chloroquine on ceasing of adherence of previously adhered cells (□). Control: 1462 ± 320 cpm ^{51}Cr in 10^6 adhered macrophages (in the case of freshly adhered cells), 2572 ± 470 cpm ^{51}Cr in 10^6 adhered macrophages (in the case of previously adhered cells).

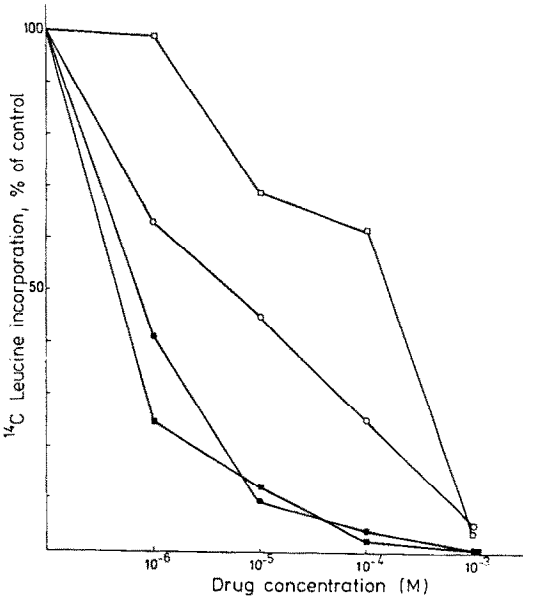

Fig. 4. Effect of drugs on the amino acid incorporation of rat peritoneal macrophages elicited by thioglycollate broth *in vivo*. Dependence on various concentrations of drugs. Symbols: pre-treatment with emetine for 60 min (●), treatment with emetine for 60 min during only amino acid incorporation without pre-treatment (■), pre-treatment with chloroquine for 60 min (○), treatment with chloroquine for 60 min only during amino acid incorporation without pre-treatment (□). Samples without pre-treatment were incubated in Eagle medium containing 10% rat serum for 60 min pre-treatment period. Control: 1565 ± 143 cpm ^{14}C-Leu per 3×10^6 cells per hour (considered as 100%).

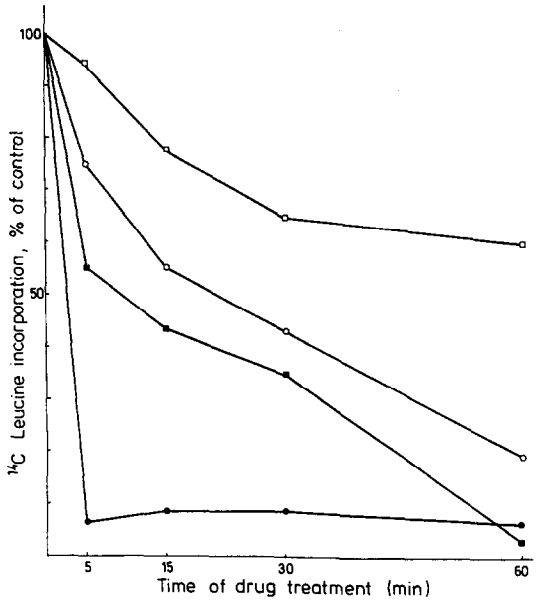

Fig. 5. Effect of drugs on the amino acid incorporation of rat peritoneal macrophages elicited by thioglycollate broth *in vivo*. Dependence on treating periods. Symbols: pre-treatment with emetine for 60 min (●), treatment with emetine during the final period of amino acid incorporation (60 min) without pre-treatment (■), pre-treatment with chloroquine for 60 min (○), treatment during the final period of amino acid incorporation (60 min) without pre-treatment (□). Control: 1823 ± 298 cpm ^{14}C-Leu per 3 × 10^6 cells per hour (considered as 100%).

to be a two-step process: the attachment is a passive event, while the anchoring is energy-dependent [21, 22]. This latter step was studied by using NaF and the dependence of the adherence on glycolysis was proven [23]. However, we mentioned above that these drugs are not known as glycolytic inhibitors. Moreover, the inhibition of phagocytosis cannot be explained merely on the basis of the decrease of adherence, e.g. at 10^{-5} M concentration of emetine the adherence was 79.6% of the control while the ingestion fell to under 40% of the control. A similar effect was observed when previously adhered macrophages were treated with the drugs (Fig. 3).

Lysosomotropic agents had no effect on the uptake of Listeria monocytogenes, but they inhibited the degradation of ingested particles [6]. In contrast, Dijkstra *et al.* [24] and Tietze *et al.* [25] found that these drugs decreased the uptake of liposomes or certain glycoconjugates. Since chloroquine has a lysosomotropic property, an indirect influence cannot be excluded, i.e. if the degradation of particles is inhibited, the ingested particles will be accumulated in the cytoplasm of macrophages resulting in the consequent blocking of the uptake.

Emetine has been known for years as an effective inhibitor of amino acid incorporation into acid insoluble fractions [10]. This inhibitory effect is more marked than the inhibition of the phagocytic process: at 10^{-6} M concentration the amino acid incorporation fell to 41% ("pre-samples") and 25% ("same samples"), while phagocytosis was diminished only to 75% ("pre") and 95% ("same") of the control (Figs. 1 and 4). Similar results were obtained with chloroquine, but the effect was not as marked as with emetine. The time dependence showed a similar pattern at least for emetine: at 5 min emetine caused a practically total breakdown in the amino acid incorporation ("pre") in contrast to a 40% inhibition of phagocytosis and a 45% inhibition of incorporation ("same") without blocking ingestion (Figs. 2 and 5). The effect of chloroquine was not so marked and the inhibition of protein synthesis and phagocytosis showed a more comparable pattern than for emetine. The irreversibility of drug treatment was demonstrated both on protein synthesis and on phagocytosis (Tables 1 and 4).

Although emetine and chloroquine inhibit both protein synthesis and phagocytosis, it is not likely to suppose a direct relationship between the two processes. Instead, one can think it to be a coincidence and the drugs must influence the contractile system of the cell's cytoskeleton. The importance of this system in phagocytosis is well known. Several authors described how pseudopods were formed on the phagocytic surface during the engulfment. The condition of this process is that the cytoskeleton be intact. The concentration of these filaments was observed on the cellular pole involved in ingestion and the gelification of actin was also described [26]. These phenomena were impaired by cytochalasin B, an agent able to destroy the microfilament system [27–29]. A limited amount of data is available about the relation between the inhibition of protein synthesis and phagocytosis. The inhibitory effect of cycloheximide and puromycine on the phagocytosis

Table 4. The irreversible effect of emetine and chloroquine on amino acid incorporation

Time after removing of the drug (min)	Amino acid incorp. with emetine (%)	Amino acid incorp. with chloroquine (%)
Control	100	100
0	2.85	12.75
30	2.44	4.32
60	0	3.09
120	1.80	2.70

Treatment with drugs was performed for 60 min. at 5 × 10^{-5} M. Control: 1666 ± 145 cpm ^{14}C-leucine per 3 × 10^6 cells per hour.

of polystyrene spheres was demonstrated by Schuit [30]. The blocking effect could not be observed during an incubation period shorter than 1 hr (see also our studies).

The cytoskeleton plays an important role both in ingestion and in adherence. One can suppose that emetine, having a hydrophobic part, can easily penetrate the membrane without destroying it and may act inside the cells both on the cytoskeleton and on ribosomes involved in protein synthesis [31, 32]. An analogous effect of cycloheximide on ribosomes in protein synthesis and on the cytoskeleton in phagocytosis may also be supposed. By all means the early inhibition of phagocytosis similar to our observations was not found with other protein synthesis inhibitors [30]. Boersma *et al.* described a protein responsible for the effect of emetine in the smaller subunit of ribosomes and they considered this protein to be a participant of the peptidyl-tRNA translocation [33], a process that requires intracellular motion similar to phagocytosis.

Taking these results into account, a direct inhibition of phagocytosis through the decrease of protein synthesis is unlikely. Rather, we may suppose that both drugs exert their effects by the impairment of the cytoskeleton leading to an irreversible reduction of ingestion, adherence and protein synthesis without influencing the attachment of particles to surface receptors and membrane-bound 5′ nucleotidase ectoenzyme activity. In the case of chloroquine, an indirect effect through the blocking of the degradation of ingested particles cannot be excluded. Lysosomotropic agents such chloroquine were described as potent inhibitors of antigen processing [34, 35] and this phenomenon also involved internalizing steps.

Finally, it must be emphasized that both emetine and chloroquine are applied in clinical use. Chloroquine is used as an antimalarial drug, while emetine is used as an effective agent against bronchitis and amoebiasis. Since our observations were based on *in vitro* experiments on rat macrophages, one should be cautious before assuming that these drugs also have an unfavourable side effect on human phagocytic processes.

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