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Effect of Glucocorticosteroids on the Phagocytosis and Intracellular Killing by Peritoneal Macrophages

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The effect of hydrocortisone on the phagocytosis and intracellular killing by mouse peritoneal macrophages in vitro was studied by a method making it possible to measure these processes separately. The results showed that in vivo treatment with 15 mg of hydrocortisone acetate did not significantly decrease the phagocytosis of several bacterial species such as *Staphylococcus albus*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa*. The killing indexes of normal macrophages for the various microorganisms were found to be significantly different. This may indicate that the bactericidal mechanisms are not uniform for these bacteria. The effect of hydrocortisone on the intracellular killing was also variable. For *Staphylococcus albus* a normal killing index was found. For the other species of bacteria and for *Candida albicans* some decrease was found, but this was only significant for *Salmonella typhimurium*. It is concluded that a decreased host resistance due to glucocorticosteroid treatment is not caused by a direct effect of these drugs on the phagocytosis and intracellular killing by mononuclear phagocytes.

Glucocorticosteroids can diminish host resistance, as indicated, for instance, by an increased susceptibility to infections (2, 13, 16, 24). It is clear that more than one factor must contribute to this effect (6, 38). One of the most important host defense mechanisms against invading microbial pathogens is phagocytosis and subsequent intracellular killing by phagocytic cells, i.e., granulocytes and mononuclear phagocytes. The available data concerning the effect of glucocorticosteroids on the functions of these phagocytic cells are conflicting (1, 9, 14, 15, 20-22, 25, 27, 31, 35).

The effect of these drugs on the phagocytic and bactericidal functions of mononuclear phagocytes has been studied mainly in intact animals, or in isolated organs, e.g., the liver, from glucocorticosteroid-treated animals. In these studies the effect could, however, be due to either an altered function of phagocytic cells or a decrease in their number. Recently, it was reported that glucocorticosteroids influence the kinetics of mononuclear phagocytes (10, 29) in several species of animals and in humans, resulting in a severe monocytopenia in the peripheral blood (7, 18, 26, 28, 30) and a decreased number of macrophages in an acute inflammatory exudate (28). Furthermore, it has also recently been shown that glucocorticosteroids interfere with the action of lymphokines in stimulating the functional activity of macrophages (3). It is, however, still uncertain whether glucocorticosteroids can effect the function of the macrophages directly, for instance by diminishing phagocytosis and/or intracellular killing of microorganisms.

Recently developed methods for the in vitro determination of these macrophage functions (12) permit assessment of the effect of glucocorticosteroids on a well-defined population of cells. The present report concerns the effect of hydrocortisone on both the phagocytosis and intracellular killing of several bacterial species and *Candida albicans* by unstimulated mouse peritoneal macrophages.

MATERIALS AND METHODS

The methods that permit separate measurement of the processes of phagocytosis and subsequent intracellular killing are described in detail elsewhere (12).

Microorganisms. The microorganisms used were *Staphylococcus aureus* (strain 421, phage type 42d), *Staphylococcus albus* (type S6, according to Baird Parker), *Pseudomonas aeruginosa* (type P_2AB, according to Vernon), *Escherichia coli* (type 073), *Salmonella typhimurium* (type 505), and *C. albicans*. The microorganisms were cultured overnight in nutrient broth (Oxoid Ltd., London), washed twice in saline, and resuspended to the required concentration in Hanks solution containing 0.1% gelatin.

Phagocytosis. Peritoneal macrophages were derived from specific-pathogen-free Swiss mice, raised in the Central Institute for the Breeding of Laboratory Animals, Bilthoven, The Netherlands. The unstimulated peritoneal cavity of these mice contains

about 80% macrophages and about 20% lymphocytes; granulocytes account for less than 1% of the cells (28). The peritoneal cells were collected with sterile phosphate-buffered saline (pH 7.2) (Difco Laboratories, Inc., Detroit, Mich.) containing 50 U of heparin per ml without conservant. Washouts collected from 20 normal mice or 25 mice treated 3 days earlier with 15 mg of hydrocortisone acetate (Merck, Sharp and Dohme, Haarlem, The Netherlands) gave a total yield of about 4×10^7 macrophages. The cells were concentrated by centrifugation for 4 min and resuspended in 2 ml of Hanks solution (Microbiological Associates, Inc., Bethesda, Md.) to which 0.1% gelatin (Difco) and 10 U of heparin per ml were added.

The opsonic serum was generally newborn calf serum (Grand Island Biological, Grand Island, N.Y.), but in the experiments with *Salmonella typhimurium* use was made of pig serum prepared from the blood of slaughterhouse animals.

For the phagocytosis measurements, 2×10^7 macrophages and 2×10^7 microorganisms were mixed. The final concentration of the opsonizing serum was 10%. In the hydrocortisone experiments, 100 μg of hydrocortisone hemisuccinate per ml (Organon, Inc., Oss, The Netherlands) was added to the medium. Siliconized glass tubes were used to prevent macrophage adherence to the wall. The bacteria-cell suspension was incubated for up to 120 min at 37 C under continuous rotation (4 rpm). After 0, 60, and 120 min a 0.5-ml sample was taken from the suspension, added immediately to 1.5 ml of ice-cold Hanks solution to stop phagocytosis, and centrifuged for 4 min at $110 \times g$ to sediment the macrophages, the unphagocytized bacteria remaining in the supernatant. (Centrifugation of a bacterial suspension at $110 \times g$ for 10 min showed that the microorganisms were not sedimented.) Serial 10-fold dilutions of the supernatant were made in buffered saline. Two duplicate 0.1-ml samples of three consecutive dilutions, with an expected number of 10^2 to 10^4 bacteria per ml, were plated on diagnostic sensitivity medium agar (Oxoid) and incubated at 37 C overnight. Plates with 10 to 500 colonies were counted with an electric colony counter, and the number of bacteria in the supernatant was calculated.

Killing. For determination of the intracellular killing of microorganisms by macrophages, independent of phagocytosis, the cells were allowed to phagocytize the microorganisms in vivo. About 2×10^6 gram-positive or 2×10^7 gram-negative bacteria, suspended in 1 ml of 10% gelatin Hanks solution with 10% opsonizing serum, were injected intraperitoneally. In the hydrocortisone experiments 100 μg of hydrocortisone hemisuccinate per ml was added to the suspension. Exactly 3 min after the injection, the mice were killed by a cervical blow and 1 min later the cells were washed out with buffered saline and collected in a tube held in crushed ice to stop further phagocytosis and to prevent the process of intracellular killing. This suspension contains about 1% granulocytes as normally found. The washouts of five mice were pooled. To remove nonphagocytized bacteria, the cells were washed twice in ice-cold gelatin-Hanks solution, centrifuged for 4 min at $110 \times g$, resuspended in 5 ml of gelatin-Hanks solution, and then divided equally over five siliconized tubes. In the hydrocortisone experiments 100 μg of hydrocortisone hemisuccinate per ml was added to the medium as well.

Four tubes were incubated at 37 C, and after 0, 15, 30, and 60 min the number of viable microorganisms within the macrophages was determined. The cells were sedimented by centrifugation for 4 min at $110 \times g$, the supernatant was discarded, and the cells were resuspended in 1 ml of ice-cold distilled water containing 0.01% bovine serum albumin (Ploviet, Amsterdam, The Netherlands). Next, the macrophages were disrupted by freezing the cell suspension in liquid air (−170 C) and thawing at 37 C three times. (Control experiments showed that the microorganisms are not killed to any appreciable degree by this freezing and thawing procedure.) In 10-fold serial dilutions made in buffered saline, the number of viable bacteria was determined as described for phagocytosis.

General remarks. The values in the figures and tables are the means of three experiments. The phagocytic index (F_t) is defined as the decrease in the number of viable bacteria in the supernatant during a given interval and calculated according to the equation $F_t = \log N_{t=0} - \log N_t$, where N is the number of viable bacteria in the supernatant and t is time. The killing index (K_t) is defined as the decrease in the number of viable intracellular microorganisms during a given interval and calculated according to the equation $K_t = \log N_{t=0} - \log N_t$, where N is the number of viable microorganisms in the macrophages and t is time. Statistical analysis of the data was done by an analysis of variance.

RESULTS

Phagocytosis. The incubation of bacteria and cells in serum-containing medium at 37 C under continuous rotation led to a decrease in the number of viable bacteria in the medium. The results of representative experiments of the phagocytosis of bacteria by peritoneal macrophages are shown in Fig. 1 and Table 1. Since this decrease could be due to several other factors besides phagocytosis, a number of control experiments were performed.

First, bacteria were incubated for 2 h in the medium without cells to make it possible to exclude a bactericidal effect of the serum and also to find out whether growth of extracellular bacteria occurred during phagocytosis. The results show that newborn calf serum had no bactericidal effect on the gram-positive bacteria and that such an effect on gram-negative bacteria could be prevented by heat inactivation of the opsonizing serum for 30 min at 56 C. Furthermore, the number of bacteria remained roughly constant over a period of 2 h except for *Salmonella typhimurium*, which showed considerable outgrowth after 60 min. On this basis, the phagocytic index was usually determined

after 120 min, but for salmonella after 60 min of incubation.

Secondly, the bacteria-cell suspension was incubated without rotation (standing control) to find out whether substances secreted by phagocytic cells or released from dying cells had bactericidal action. No decrease in the number of viable extracellular bacteria occurred (Fig. 1). This experiment also demonstrated that to achieve a measurable degree of phagocytosis, it is necessary to rotate the suspensions continuously.

Thirdly, bacteria and cells were also incubated in medium without serum. Under this condition no phagocytosis occurred; the presence of serum proved to be necessary for opsonization of bacteria. This experiment also showed that newborn calf serum did not contain opsonizing factors for *Salmonella typhimurium*, whereas pig serum opsonized these bacteria adequately. Inactivation of the sera by heating at 56 C did not abolish the opsonizing effect in the gram-negative bacteria.

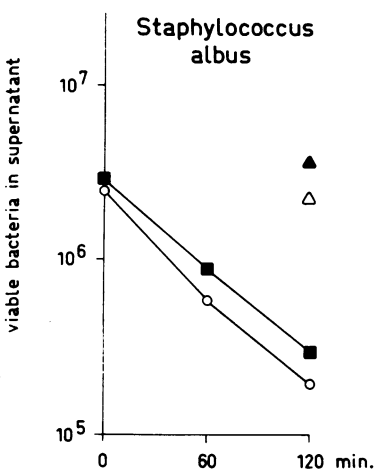

FIG. 1. *In vitro phagocytosis by peritoneal macrophages. Decrease in the number of viable bacteria in the supernatant caused by ingestion of the microorganisms by the macrophages during continuous rotation. The stationary sample served as control to show that the decrease was not due to bactericidal effect of the incubation medium. Symbols:* (○) *Normal mice;* (■) *hydrocortisone-treated mice;* (▲, △) *standing controls.*

The in vitro phagocytosis of *Candida* could not be assessed because these organisms and macrophages have about the same sedimentation velocity and therefore could not be separated.

Effect of hydrocortisone on the phagocytosis of bacteria. The effect of hydrocortisone on phagocytosis by peritoneal macrophages was determined in cells taken from mice injected subcutaneously 72 h earlier with a depot of 15 mg hydrocortisone acetate. This dose causes only a slight decrease in the total number of macrophages in the peritoneal cavity after 3 days (28).

The results of these experiments are given in Fig. 1 and Table 1. *Staphylococcus albus* and *E. coli* showed a slight but not significant decrease in phagocytosis, but a significantly diminished phagocytic index was found for *P. aeruginosa* ($P < 0.01$).

Since this decrease could be due to either the hydrocortisone treatment of the mice or the hydrocortisone added in vitro, or a combination of both, the effect of each of these modes of administration was determined separately (Table 2). Macrophages from hydrocortisone-treated mice in a hydrocortisone-free medium phagocytized normally, and macrophages from untreated mice ingested pseudomonas to a significantly lower degree in the presence than in the absence of hydrocortisone hemisuccinate. When 1 or 10 μg of hydrocortisone hemisuccinate per ml was added in vitro, this effect was not found. A bactericidal effect of hydrocortisone could be excluded, since the incubation of the bacteria in medium with 100 μg of hydrocortisone per ml for 2 h gave no reduction of the number of viable bacteria. These results lead to the conclusion that the decreased phagocytosis of pseudomonas is an in vitro effect of hydrocortisone hemisuccinate. Whether this effect is due to the interference of hydrocortisone with the attachment to or ingestion of pseudomonas by the phagocytic cells deserves further investigation.

TABLE 1. *Effect of hydrocortisone on phagocytosis by peritoneal macrophages*[a]

Macrophages	*Staphylococcus albus* (F_{120})	*Staphylococcus aureus* (F_{120})	*Pseudomonas aeruginosa* (F_{120})	*Escherichia coli* (F_{120})	*Salmonella typhimurium* (F_{60})
Normal	1.1247	0.4469	1.1162[b]	0.3966	0.2565
From hydrocortisone-treated animals	0.9649	0.4905	0.6973[b]	0.2376	0.2953

[a] Expressed as $F_t = \log N_{t=0} - \log N_t$.
[b] $P < 0.01$.

Intracellular killings. For reliable measurement of the intracellular killing, the macrophages must contain a sufficient number of viable microorganisms at the beginning of assay. Under our conditions the rate of in vitro phagocytosis by macrophages was too low in relation to the rate of intracellular killing to meet this condition (unpublished observation). When the macrophages are allowed to phagocytize the microorganisms in vivo, however, sufficient bacteria are ingested in a very short time to permit subsequent measurement of intracellular killing. Microscopically, it was found that after 4 min about 42% of the macrophages had ingested two or more *Staphylococcus albus*, giving a mean number of 3.1 bacteria per cell. For *C. albicans*, about 75% of the macrophages had ingested one or more yeasts, with a mean of 1.4 microorganisms per macrophage; only a few *Candida* were found extracellularly.

The results of the measurement of intracellular killing are given in Table 3 and representative experiments are illustrated in Fig. 2. The decrease in the number of viable intracellular microorganisms could only be measured over a period of 60 min, since thereafter growth of the remaining viable microorganisms made further assessment of the killing process impossible. The sample incubated at 4 C showed that the decrease in the number of viable microorganisms was due to an active intracellular process.

TABLE 2. *In vivo and in vitro effects of hydrocortisone on the phagocytosis of Pseudomonas aeruginosa by peritoneal macrophages*[a]

Medium	Macrophages	
	Normal	From hydrocortisone-treated mice[b]
Without hydrocortisone	1.1162[c]	1.0495
With hydrocortisone[d]	0.7993[c]	0.6973

[a] Expressed as $F_{120} = \log N_{t=0} - \log N_{120}$.
[b] 15 mg of hydrocortisone acetate subcutaneously.
[c] $P < 0.01$.
[d] Hydrocortisone hemisuccinate, 100 μg/ml in medium.

Effect of hydrocortisone on the intracellular killing of microorganisms. The effect of hydrocortisone on the intracellular killing of microorganisms by peritoneal macrophages was determined in cells from mice treated with 15 mg of hydrocortisone acetate 72 h previously (Fig. 2 and Table 3). For *Staphylococcus albus*, no decrease was found. Although for *Staphylococcus aureus*, *C. albicans*, and all gram-negative microorganisms the killing was on a lower level during glucocorticosteroid treatment, a significantly diminished ($P < 0.01$) killing was only found for *Salmonella typhimurium*.

To determine whether this decrease was an in vivo or in vitro glucocorticosteroid effect, the effect of each mode of administration was determined separately (Table 4). The decreased intracellular killing of *Salmonella typhimurium* proved to be due to the in vivo treatment of mice with glucocorticosteroids.

DISCUSSION

The present study demonstrates that treatment of mice with hydrocortisone has little effect on the phagocytosis and intracellular killing of several bacterial species by noninduced peritoneal macrophages. Only the intracellular killing of *Salmonella typhimurium* is significantly decreased.

The lack of an effect of hydrocortisone treatment on bacterial killing was contrary to our expectation that stabilization of lysosomal membranes, as shown for liver lysosomes and artificial liposomes (33), could lead to a decreased bactericidal activity of the macrophages due to impaired fusion of the lysosomes with the phagocytic vacuole. In an electron microscopic study, the lysosome-phagosome fusion was observed to be normal (11).

Another striking result was that the killing indexes of macrophages of normal mice for various species of microorganisms were significantly different ($P < 0.01$). This may be an indication that macrophage bactericidal mechanisms are not uniform for all microorganisms,

TABLE 3. *Effect of hydrocortisone on intracellular killing by peritoneal macrophages*[a]

Macrophages	*Staphylococcus albus*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Salmonella typhimurium*	*Candida albicans*
Normal	1.0747	1.1342	1.3174	0.6276	0.7476[b]	0.6427
From hydrocortisone-treated animals	1.2039	1.0539	1.0420	0.4789	0.4961[b]	0.4611

[a] Expressed as $K_{60} = \log N_0 - \log N_{60}$.
[b] $P < 0.01$.

which in turn may account for different effects of steroid treatment. It must be kept in mind, however, that the biochemical events responsible for intracellular killing of microorganisms by macrophages are still mainly unknown (17). Therefore, the explanation of a reduced microbicidal action on the basis of processes such as lysosomal stabilization or an impaired reduced nicotinamide adenine dinucleotide function (20) after steroid treatment remains speculative.

The results of the present study agree with the studies of McCall et al. (19) and Wang et al. (32), who found that in unstimulated alveolar macrophages biochemical events occurring during phagocytosis were unaltered by methylprednisolone. Only the stimulation of these macrophage functions by BCG vaccination was suppressed. Balow and Rosenthal (3) found, in a study on the target point of suppression of cellular immunity by glucocorticosteroids, that pretreatment of macrophages with hydrocortisone did not result in a diminished uptake or processing of antigen. However, activation of the macrophages by migration inhibition factor (MIF), reflected in an increased migration inhibition, could be blocked by hydrocortisone. Since MIF can also induce an increased bactericidal activity of macrophages (8, 23), blocking of the MIF-macrophage interaction by glucocorticosteroids could result in diminished intracellular killing of microorganisms.

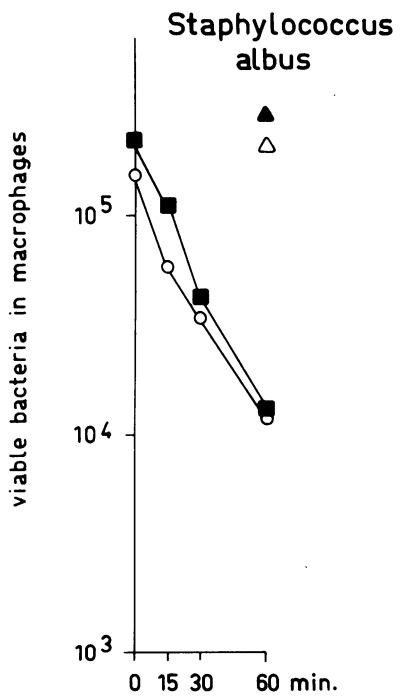

FIG. 2. *In vitro intracellular killing of bacteria by peritoneal macrophages. Decrease in the number of viable bacteria caused by intracellular killing of the microorganisms at 37 C after phagocytosis in vivo. The sample incubated at 4 C served as control to demonstrate that the intracellular death was due to a metabolic active process. Symbols:* (○) *Normal mice;* (■) *hydrocortisone-treated mice;* (▲, △) *incubation at 4 C.*

TABLE 4. *In vivo and in vitro effects of hydrocortisone on the intracellular killing of Salmonella typhimurium by peritoneal macrophages*[a]

Medium	Macrophages	
	Normal	From hydrocortisone-treated mice[b]
Without hydrocortisone	0.7476[c]	0.4345[c]
With hydrocortisone[d]	0.8236	0.4961

[a] Expressed as $K_{60} = \log N_{t=0} - \log N_{60}$.
[b] 15 mg of hydrocortisone acetate subcutaneously.
[c] $P < 0.01$.
[d] 100 μg of hydrocortisone hemisuccinate per ml.

In contrast to these findings, Wiener et al. (37) found a decreased in vitro uptake of aggregated human serum albumin and *Salmonella typhimurium* by macrophage obtained from normal animals in the presence of hydrocortisone. It should be noted, however, that the most marked effect was observed with a hydrocortisone concentration 10 times higher than was used in this study, and it is not clear from their data whether the decrease observed with a dose comparable to that used in the present study was statistically significant. The subsequent degradation of the ingested human serum albumin was found to be unaltered by hydrocortisone. In another study these authors did not observe an effect of hydrocortisone on the pinocytic activity of cultured macrophages (36).

Hydrocortisone treatment of the mice caused a significantly decreased killing only of *Salmonella typhimurium*, although some decrease was also found for *Staphylococcus aureus*, *P. aeruginosa*, *E. coli*, and *C. albicans*. The reason for this difference in the effect is unclear. If hydrocortisone interfered directly with the bactericidal mechanism of the macrophages, this effect could be expected to be the same for all of the microorganisms investigated, which was not the case. Alternatively, it could be argued that hydrocortisone treatment alters the functional state of the macrophages in relation to *Salmonella*. Although the macrophages used in this study were unstimulated and had not been

induced, this does not necessarily mean that such cells are in a zero-functional state with respect to all microbial pathogens. However, since the macrophages were obtained from a specific-pathogen-free strain of mice, it is hardly likely that these animals were sensitized for salmonellas.

From the present findings it may be concluded that a direct effect of gluococorticosteroids cannot account for a grossly diminished phagocytosis or intracellular killing of microorganisms by mononuclear phagocytes. This does not mean that glucocorticosteroid treatment cannot lead to decreased host resistance as a result of an altered number and/or function of the mononuclear phagocytes at the site of the infections. Glucocorticosteroids suppress the recruitment of monocytes from the bone marrow at the site of infection (10, 29), leaving fewer macrophages available for the elimination of microorganisms (28). In addition, a suppression of cell-mediated immunity could lead to a deficient functional state of the macrophages (3–5, 34). Earlier publications in which a diminished function of macrophages after glucocorticosteroid treatment was reported on the basis of studies on the clearance of bacteria or particular matter in intact animals or in isolated organs (31) must be interpreted in terms of these mechanisms. Thus a decreased mobilization and/or immunological stimulation of mononuclear phagocytes, but not a direct effect of glucocorticosteroids on these cells, may contribute to impaired host resistance during glucocorticosteroid treatment.

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