

# $\gamma$ -Interferon Restores Listericidal Activity and Concurrently Enhances Release of Reactive Oxygen Metabolites in Dexamethasone-treated Human Monocytes

Andreas Schaffner and Philipp Rellstab

Department of Medicine, Medical Clinic, University of Zürich Medical School, CH-8091 Zürich, Switzerland

## Abstract

By preventing macrophages from destroying phagocytized microorganisms, glucocorticoids lower natural resistance of the host against many pathogens.  $\gamma$ -Interferon has an opposite effect and restores activity of dexamethasone-treated mononuclear phagocytes against some but not all microorganisms. In the present studies we show that dexamethasone impairs activity of human blood monocytes kept in culture for 36 h against *Listeria monocytogenes*, without impairing  $H_2O_2$  or  $O_2^-$  secretion. Likewise dexamethasone does not interfere with activation of systems generating oxygen metabolites by lymphokines. Thus  $\gamma$ -interferon increases three- to fivefold the capacity of dexamethasone-treated monocytes to secrete  $O_2^-$  or  $H_2O_2$  upon stimulation by opsonized zymosan, live bacteria, or phorbol myristate acetate. Concurrently  $\gamma$ -interferon restores listericidal activity of dexamethasone-treated monocytes. After gradual activation by exposure to  $\gamma$ -interferon for progressive time periods, listericidal activity becomes tightly correlated ( $r = 0.922-0.994$ ) with the amount of  $H_2O_2$  or  $O_2^-$  secreted by dexamethasone-treated monocytes. Activation of oxidative systems by the lymphokine is, however, not correlated with the restoration of activity against *Aspergillus* spores, lost during dexamethasone treatment, which does not depend on antimicrobial oxygen metabolites. Taken together, these observations lend to the hypothesis that glucocorticoids impair nonoxidative defense mechanisms of "resting" macrophages, while  $\gamma$ -interferon restores macrophage function impaired by glucocorticoids by activating alternate killing systems, which are at least partly of oxidative nature.

## Introduction

Glucocorticoids impair the antimicrobial activity of mononuclear phagocytes and affect by this mechanism natural immunity against bacteria and fungi in several models of opportunistic infection (1-4). Glucocorticoids impair antimicrobial function by a direct hormonal effect on the phagocyte and prevent macrophages from killing and eliminating ingested microorganisms (2, 3, 5). In contrast, lymphokines such as  $\gamma$ -interferon enhance the antimicrobial activity of macrophages (5-7) and increase their capacity to secrete reactive oxygen intermediates (ROI)<sup>1</sup> (6). We have previously reported

Address reprint requests to Dr. Schaffner.

Received for publication 6 October 1987 and in revised form 4 March 1988.

1. Abbreviations used in this paper: HRP, horseradish peroxidase; KRPG, Krebs-Ringer phosphate glucose; ROI, reactive oxygen intermediates.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/88/09/0913/07 \$2.00

Volume 82, September 1988, 913-919

that  $\gamma$ -interferon restores the antimicrobial activity of human blood-derived macrophages exposed to glucocorticoids against *Listeria monocytogenes* and *Salmonella typhimurium*, two facultative intracellular bacteria susceptible to ROI, but not against microorganisms more resistant to ROI, such as *Nocardia asteroides* and *Aspergillus* spores (5). We therefore speculated that glucocorticoids impair nonoxidative killing systems of mononuclear phagocytes operative in resting macrophages. Accordingly  $\gamma$ -interferon by activating oxidative killing systems would overcome the functional impairment by glucocorticoids of resting cells and restore activity of macrophages against microorganisms susceptible to oxidants.

By investigating the effects of glucocorticoids and of  $\gamma$ -interferon on secretion of ROI in relation to antimicrobial activity in human blood monocytes, we confirm in the present studies that glucocorticoids impair nonoxidative killing systems, operative in nonactivated, "resting" mononuclear phagocytes. After impairment of these antimicrobial functions by dexamethasone, activation of oxidative systems by  $\gamma$ -interferon becomes tightly correlated to activity against *Listeria monocytogenes* but not to activity against *Aspergillus* spores, known to be independent from ROI. The correlation of ROI secretion with antimicrobial activity in human blood-derived macrophages simultaneously exposed to dexamethasone and  $\gamma$ -interferon permits visualization of a distinct role in host defense for nonoxidative killing systems operative in "resting" mononuclear phagocytes, and of other killing systems presumably at least in part of oxidative nature, operative in the activated state of the phagocyte.

## Methods

**Organisms.** A lot of the *Listeria monocytogenes* strain EGD kept virulent by passage through mice was frozen in mid-log phase in trypticase soy broth at  $-70^\circ\text{C}$ . For each experiment *Listeria* was grown as standing culture in trypticase soy broth at  $37^\circ\text{C}$  overnight giving  $1.2-1.9 \times 10^9$  CFU. Bacteria were washed three times in Gey's balanced salt solution, opsonized for 20 min in fresh human serum at  $37^\circ\text{C}$ , and washed once more before dilution (1:12 to 1:15) in medium supplemented with 30% serum for infection of macrophages. This gave suspensions with a uniform cell count of  $1-1.5 \times 10^8$  CFU of bacteria per ml. Single-spore suspensions from a clinical isolate of *Aspergillus fumigatus* were obtained as described previously (5).

**Media, serum, and reagents.** Trypticase soy broth and trypticase soy agar were from Difco Laboratories, Detroit, MI. Medium-199 (M-199) and Gey's balanced salt solution were from Gibco Europe, Basel, Switzerland. Pyrogen-free human recombinant  $\gamma$ -interferon was a gift from Biogen SA, Geneva, Switzerland. Dexamethasone, luminol, lucigenin, scopoletin, zymosan, phorbol myristate acetate (PMA), heparin, superoxide dismutase (type I), catalase (from bovine liver), horseradish peroxidase (type IV) (HRP), and bovine serum albumin were all from Sigma Chemical Co., St. Louis, MO. Ficoll-Hypaque was from Pharmacia, Inc., Piscataway, NJ. Serum from normal volunteers was prepared as described (5).

**Human blood-derived macrophages.** All studies were performed on human monocytes kept in culture for 36-48 h. Blood mononuclear

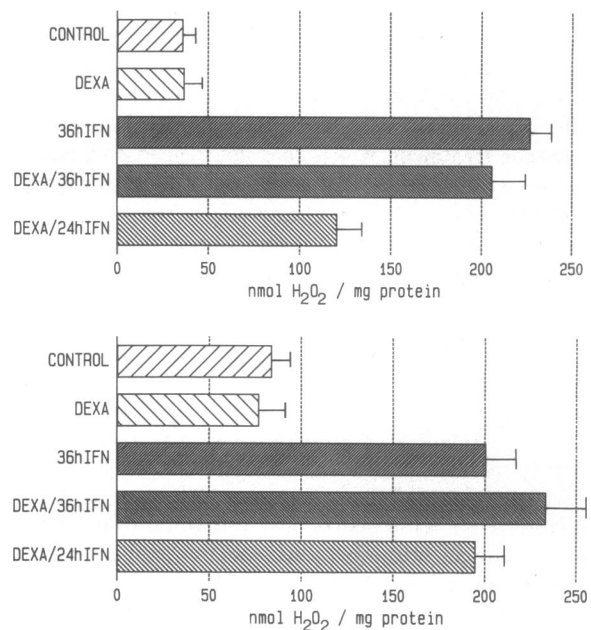
cells from normal volunteers were separated by the Ficoll-Hypaque technique and monolayers were prepared on round 9- or 12-mm glass coverslips by a slight modification of the technique of Nagakawara et al. (8) as described previously (9). In brief, 100  $\mu$ l (for 12-mm coverslips) or 50  $\mu$ l (for 9-mm coverslips) of a suspension of  $1.5 \times 10^7$  washed mononuclear cells in M-199 supplemented with 25% human serum was placed on coverslips in 35-mm tissue culture dishes (Falcon Plastics, Oxnard, CA). After incubation for 1 h at 37°C for adhesion, coverslips were washed four times with prewarmed Gey's balanced salt solution and transferred into 16-mm diam plastic cluster plates (Falcon Plastics) and cultured in M-199 supplemented with 25% human serum and with additives or solvents (i.e., 0.01% of ethanol for dexamethasone, 0.1% phosphate-buffered saline for  $\gamma$ -interferon) as indicated. Monolayers always consisted of  $\geq 98\%$  mononuclear phagocytes as determined by Giemsa stain and phagocytosis of *Aspergillus* spores (5). Quantification of cell numbers from individual coverslips was done after exposure of monolayers to Zaponin (Coulter Electronics, Inc., Hialeah, FL) for stromatolysis and counting the freed nuclei in a model S counter (Coulter Electronics, Inc.). Cellular protein was measured by the method of Lowry (10), after solubilizing monolayers in 0.33 M NaOH, using bovine serum albumin as standard.

**Quantification of production of ROI by macrophages.** Secretion of  $H_2O_2$  was studied by a modification of the method of Root et al. (11) which quantifies the loss of fluorescence of scopoletin resulting from oxidation by  $H_2O_2$  in the presence of HRP. In brief, coverslips with monolayers of 40,000–160,000 mononuclear cells were washed three times in Krebs-Ringer phosphate glucose (KRP:G: 137, 8 mM NaCl, 4.8 mM KCl, 0.8 mM  $CaCl_2$ , 0.12 mM  $MgSO_4$ , 5.5 mM glucose, pH 7.38), placed in individual wells of 16-mm cluster plates in 1 ml of KRP:G supplemented with 50 nM scopoletin and 1 U/ml of HRP, and incubated in the dark at 37°C for 10 min in a water bath for warmup. Then PMA (50 ng/ml final concentration) or zymosan (100  $\mu$ l of a suspension of 5 mg/ml of preswollen, opsonized, 90% fresh human serum), and washed zymosan were added to each test well to trigger a respiratory burst. Plates with test wells, wells with monolayers to which no triggering substance was added to measure baseline  $H_2O_2$  secretion, and wells with no cells to measure spontaneous loss of fluorescence were then incubated for 1 h at 37°C in the dark, before stopping the burst by placing the trays on ice and immediately measuring loss of fluorescence on a Perkin Elmer LS-5 fluorimeter (Perkin Elmer, Inc. Küssnacht, Switzerland). Under these conditions spontaneous  $H_2O_2$  secretion and spontaneous oxidation of scopoletin were insignificant. Results are given as nanomoles of  $H_2O_2$  per milligram of cell protein secreted during 1 h from quadruplicate wells.

**Measurement of  $H_2O_2$  and  $O_2^-$  secretion by chemiluminescence.**  $H_2O_2$  secretion was quantified by chemiluminescence in a luminol-amplified system (12, 13) in an LKB 1251 luminometer (LKB Produkter, Bromma, Sweden), which converts photons by means of a photon multiplier tube into millivolts. This luminometer permits automated, simultaneous, discontinuous measurement of up to 25 samples kept at 37°C with intermittent shaking. In the presence of a peroxidase such as HRP, luminol-amplified chemiluminescence primarily measures  $H_2O_2$  with only a minimal effect of other ROI generated during the respiratory burst as shown previously by Müller-Peddinghaus (14). Accordingly, luminescence was reduced also in our system by  $> 90\%$  by omitting HRP or adding  $10^{-5}$  M  $NaN_3$  and reduced by  $> 70\%$  by adding 3,300 U/ml of catalase, but not affected or even minimally enhanced by adding 280 U of superoxide dismutase per ml. For the assay luminol was dissolved in dimethylsulfoxide (1 mg in 20  $\mu$ l) and directly diluted in KRP:G at 20°C under continuous agitation to the final concentration of  $1 \times 10^{-4}$  M. The solution was then supplemented with 1 U/ml of HRP. Coverslips with monolayers were washed by three passages through beakers with KRP:G, placed individually in cuvettes, and immediately covered with 1 ml of luminol-HRP in KRP:G. After warming the cuvettes to 37°C for 15 min, baseline chemiluminescence was recorded, the triggering substance, 50 ng/ml of PMA (final concentration) or 100  $\mu$ l of opsonized zymosan (5 mg/ml), was added, and the respiratory burst was recorded over the

next 15–30 min.  $O_2^-$  secretion was measured accordingly by lucigenin-amplified chemiluminescence (15), in which the emitted light predominantly results from oxidation by  $O_2^-$  (14). Lucigenin-amplified luminescence gave a lower yield of photons, which was suppressed by superoxide dismutase, but not by  $10^{-4}$  M  $NaN_3$  or catalase and not enhanced by HRP. Lucigenin-amplified chemiluminescence because of its insensibility to catalase permitted us to measure, in addition to a response to PMA or zymosan, a response to opsonized (20 min, 37°C, 90% fresh human serum), washed *Listeria* ( $100 \mu$ l  $10^9$  CFU/ml). Lucigenin  $1 \times 10^{-4}$  M was directly dissolved in KRP:G. Results from experiments are given as millivolts per  $10^5$  cells from at least triplicate wells.

**Antilisterial activity.** Morphologic assessment of the antilisterial activity by monitoring the percentage of infected cells was done as described previously (5). Because 7–8 h are mandatory after phagocytosis for macrophages to digest bacteria and reduce the number of cells with visible bacteria, experiments that study the effect of short-term exposure to  $\gamma$ -interferon are hindered in this assay. We therefore devised an assay that measures the bactericidal activity of macrophages after only 3 h of incubation, by following the number of bacterial CFU. Monolayers on 12-mm coverslips in 16-mm cluster wells were challenged with  $1.2$ – $1.8 \times 10^8$  opsonized (90% fresh human serum), washed *Listeria*. After incubation for phagocytosis (15 min, 37°C), noningested bacteria were removed by five washes of warm Gey's balanced salt solution, and coverslips were transferred to individual 10-ml Polypropylene test tubes (Falcon Plastics) with 1 ml of M-199



**Figure 1.** Release of  $H_2O_2$  by monolayers of human blood monocytes kept in culture for 36 h in response to PMA (upper panel) or opsonized zymosan particles (lower panel). The results are given as nanomoles of  $H_2O_2$  released during 60 min measured by quantitating the loss of fluorescence of scopoletin. The protein content was measured after solubilizing monolayers with 0.33 N NaOH by the method of Lowry et al. (10). Mean  $\pm$  SEM from four (upper) and three (lower) independent experiments with quadruplicate wells in each group and experiment. (DEXA) Exposure of monocytes to  $2.5 \times 10^{-7}$  M of dexamethasone for the whole duration of the experiment which was 36 hours. (IFN) Exposure of macrophages to 50 U/ml of human recombinant  $\gamma$ -interferon for 36 h (36hIFN) or 24 hours (24hIFN) before the scopoletin assay. Differences between  $H_2O_2$  release by monolayers exposed to dexamethasone or to solvent control (0.001% ethanol):NS. Differences between IFN and all controls:  $P < 0.001$ .

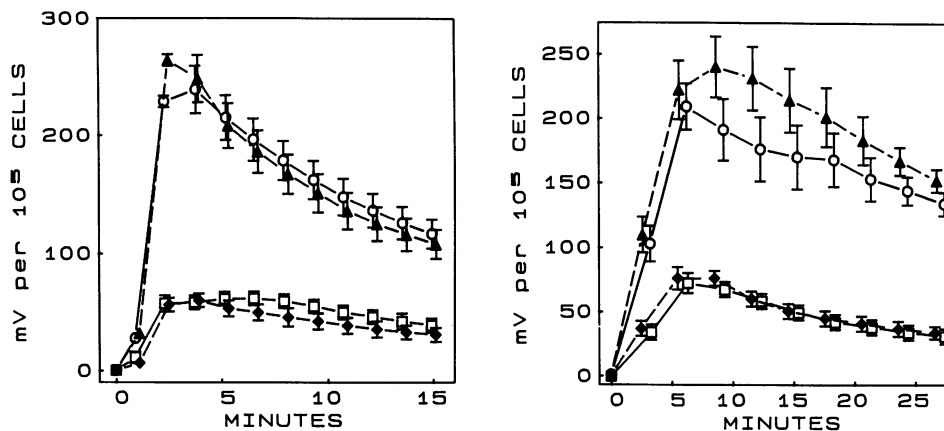


Figure 2. Luminol-HRP-amplified chemiluminescence of monolayers in response to PMA (left panel) or opsonized zymosan particles (right panel). In agreement with the results obtained with the classical assay for quantitation of H<sub>2</sub>O<sub>2</sub> secretion shown in Fig. 1, chemiluminescence in the luminol-HRP-amplified system, which principally reflects H<sub>2</sub>O<sub>2</sub> release, is identical for control cells (□) and dexamethasone-exposed cells (♦). Activation of macrophages with 50 U/ml of gamma-interferon (○) is not hindered by the simultaneous exposure to 2.5 × 10<sup>-7</sup> M of dexamethasone for 36 hours (▲). Each panel gives the results from a typical independent experiment (out of three) with quadruplicate wells, mean ± SD.

supplemented with 25% human serum. Subsequently (for baseline counts), and after incubation for 3 h, sets of four tubes were placed on ice, cells were lysed with ice-cold 0.1% deoxycholate (final concentration) in distilled water for exactly 15 min and serial dilutions of the lysate cultured on trypticase soy agar for determination of CFU (3). Under these conditions deoxycholate had no effect on the viability of *Listeria*. Results of the assay are given as the change in CFU in percentage of the baseline values after incubation of bacteria and macrophages for 3 h after phagocytosis from quadruplicate tubes.

**Statistical analysis.** Values are given as mean ± SD or ± SEM as indicated. The relation between killing of bacteria and the respiratory burst was evaluated by simple linear regression (16). Mean values were compared by *t* test (16).

## Results

**Effects of dexamethasone,  $\gamma$ -interferon, and dexamethasone plus  $\gamma$ -interferon on secretion of ROI by human blood monocytes kept in culture for 36 h.** Exposure of blood-derived macrophages to high pharmacologic concentrations of dexamethasone for 36 h, a regimen shown previously to abolish antimicrobial activity (5), had no effect on the amount of H<sub>2</sub>O<sub>2</sub> secreted by "resting" macrophages upon stimulation by PMA or opsonized zymosan as determined in an endpoint assay (Fig. 1). Dexamethasone did also not interfere with the activation of systems responsible for increased secretion of H<sub>2</sub>O<sub>2</sub> by

lymphokines as shown by a three to fivefold increase in the amount of H<sub>2</sub>O<sub>2</sub> secreted by cells activated in the continuous presence of 2.5 × 10<sup>-7</sup> M of dexamethasone (Fig. 1). Comparable results were obtained by measuring H<sub>2</sub>O<sub>2</sub> secretion by chemiluminescence amplified by peroxidase and luminol (Fig. 2), which permitted us to evaluate individual monolayers with as little as 4 × 10<sup>4</sup> cells. The observation that activation of macrophages occurs in the presence of dexamethasone was further confirmed by measuring secretion of O<sub>2</sub><sup>-</sup> by lucigenin-amplified chemiluminescence (Fig. 3). This system, in which chemiluminescence is not lessened by catalase, permitted us to investigate, in addition, secretion of ROI induced by phagocytosis of live *Listeria*, an intracellular pathogen with high catalase activity. We were particularly interested in chemiluminescence directly induced by *Listeria*, because we have reported that *Listeria* is killed by resting blood-derived macrophages but not by resting mononuclear phagocytes exposed to dexamethasone. Furthermore, we had found that the listericidal activity of macrophages activated by  $\gamma$ -interferon was not diminished by dexamethasone (5). Besides we had found a similar impairment of antimicrobial activity by dexamethasone in blood-derived macrophages from children with chronic granulomatous disease, which are unable to secrete important amounts of ROI, and therefore had speculated (5) that dexamethasone interfered with nonoxidative killing of *Listeria* by

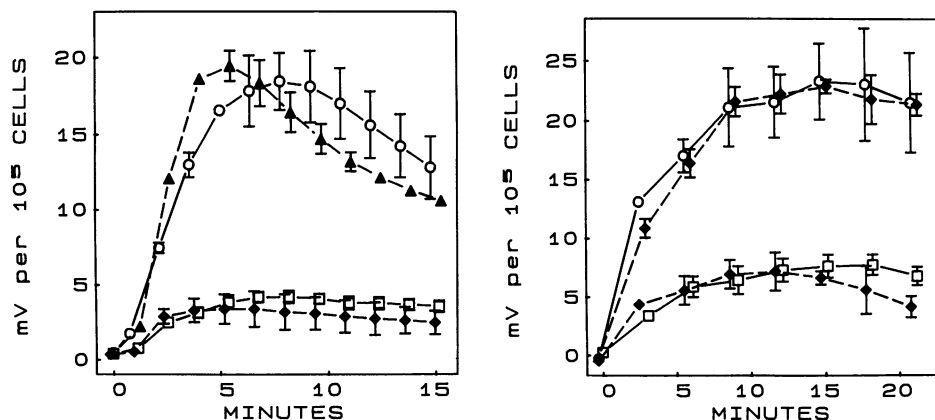
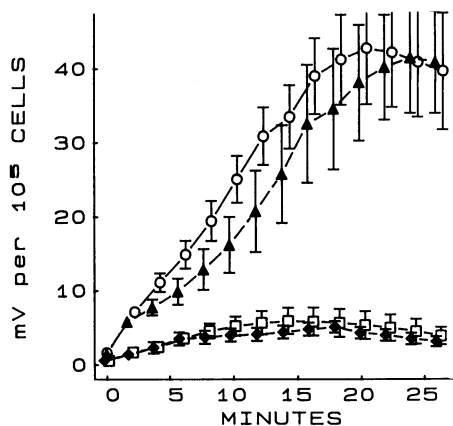


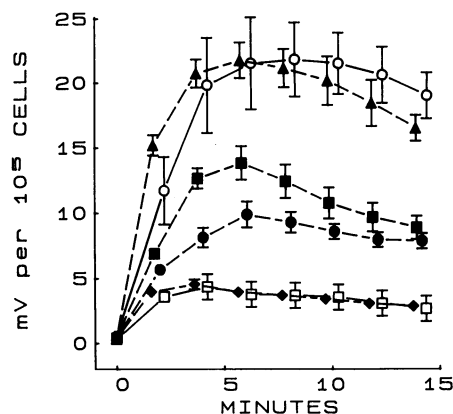
Figure 3. Lucigenin-amplified chemiluminescence in response to stimulation with PMA (left panel) or zymosan (right panel). The yield of photons was uniformly lower compared to the luminol-amplified system (Fig. 2) in the lucigenin system which principally measures O<sub>2</sub><sup>-</sup> release (see Methods). (□) Control monolayers; (♦) 2.5 × 10<sup>-7</sup> M of dexamethasone for 36 h; (○)  $\gamma$ -interferon 50 U/ml for 36 hours; (▲)  $\gamma$ -interferon plus dexamethasone for 36 h. Mean ± SD from one typical experiment out of two.



**Figure 4.** Lucigenin-amplified chemiluminescence in response to opsonized live *Listeria monocytogenes*. Comparable to the results obtained with PMA or opsonized Zymosan particles, live bacteria trigger a release of an only modest amount of  $O_2^-$  in resting macrophages not activated by lymphokines ( $\square$ ), which is not influenced by  $2.5 \times 10^{-7}$  M of dexamethasone ( $\blacksquare$ ). Activation of oxidative systems by 50 U/ml of  $\gamma$ -interferon ( $\circ$ ) is not hindered by dexamethasone ( $\blacktriangle$ ). Mean  $\pm$ SD from quadruplicate wells from one typical experiment out of two.

resting macrophages but that dexamethasone did not prevent activation of listericidal systems operative in macrophages exposed to  $\gamma$ -interferon which were probably oxidative in nature (5). In accordance with this hypothesis, we now found that *Listeria* induced secretion of a modest amount of  $O_2^-$  in resting macrophages which was not lessened by dexamethasone and that exposure of macrophages to  $\gamma$ -interferon resulted in a three- to fourfold increase in the amount of  $O_2^-$  secreted in response to a challenge with live *Listeria*, whether macrophages were exposed to dexamethasone or not (Fig. 4).

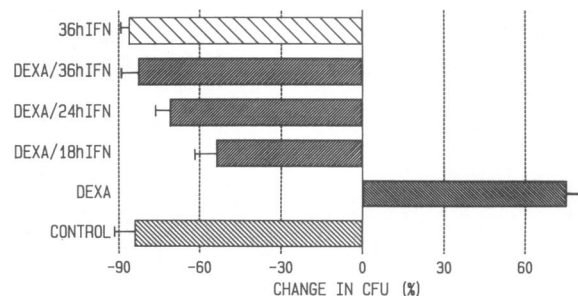
We next turned to the problem of analyzing quantitatively the correlation between ROI secreted by macrophages with listericidal activity. In preliminary experiments we found that exposure of macrophages to graded concentrations of  $\gamma$ -interferon for 36 h did not reproducibly result in accordingly graded increments in the amount of secreted ROI, presumably because of the steep dose-response curve with a barely detectable activity at 1–2 U/ml and maximal activity at concentrations between 5 and 10 U/ml (data not shown). Therefore we elected to expose macrophages for progressively longer time spans to a fully activating concentration of  $\gamma$ -interferon in order to obtain monolayers secreting progressively increasing amounts of ROI (Figs. 1 and 5). Moreover, exposure of dexamethasone-treated macrophages to  $\gamma$ -interferon for progressively longer time spans resulted in an increasingly higher listericidal activity in a test system based on quantitative cultures measuring the change in CFU of *Listeria monocytogenes* associated to macrophage monolayers after vigorous washing over time (Fig. 6).  $\gamma$ -Interferon, dexamethasone, and a combination of  $\gamma$ -interferon and dexamethasone had no significant effect on the number of bacteria remaining associated with monolayers after incubation for phagocytosis for 15 min and washing (mean  $\log_{10}$  CFU  $\pm$ SEM after washing from five independent experiments with four wells: control  $4.87 \pm 0.12$ ; dexamethasone  $5.12 \pm 0.21$ ;  $\gamma$ -interferon  $4.93 \pm 0.29$ ;  $\gamma$ -interferon plus dexamethasone  $5.01 \pm 0.15$ ; all values NS vs. control). Furthermore, it was ascertained that this assay, in which no



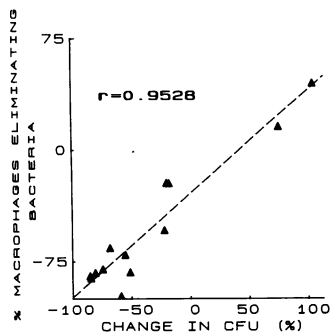
**Figure 5.** Time-response curve for  $\gamma$ -interferon induced activation of  $O_2^-$  release (lucigenin-amplified chemiluminescence) in response to PMA. Blood-derived macrophages were cultured for 36 hours in the presence of  $2.5 \times 10^{-7}$  M of dexamethasone ( $\blacklozenge$ ,  $\bullet$ ,  $\blacksquare$ ,  $\blacktriangle$ ) and simultaneously exposed to 50 U/ml  $\gamma$ -interferon for 36 h ( $\blacktriangle$ ), the last 24 h ( $\blacksquare$ ), the last 12 h ( $\bullet$ ), or 0 hours ( $\blacklozenge$ ). ( $\square$ ) Control cells, ( $\circ$ ) cells activated with 50 U/ml of  $\gamma$ -interferon in the absence of dexamethasone. Mean  $\pm$ SD from quadruplicate wells from one typical experiment out of three.

antibiotics are added to the culture medium, measured activity of macrophages directed against ingested *Listeria*. As shown by the tight relation between the change in CFU with the fate of intracellular *Listeria* followed visually over time by counting the number of parasitized macrophages, the results of the assay depending on quantitative cultures were not blurred by growth of extracellular bacteria (Fig. 7). Additionally, the comparison of the two assays also excludes the possibility that the main antilisterial activity would go unnoticed in the CFU-type assay during initial incubation for 15 min for phagocytosis, before washing and determination of the baseline values, because digestion of bacteria requires several hours and dead bacteria would be noticed in the morphological assay and lead to results deviating between the two assay systems.

This background permitted us to proceed to a direct correlation of antilisterial activity of monocytes with secretion of

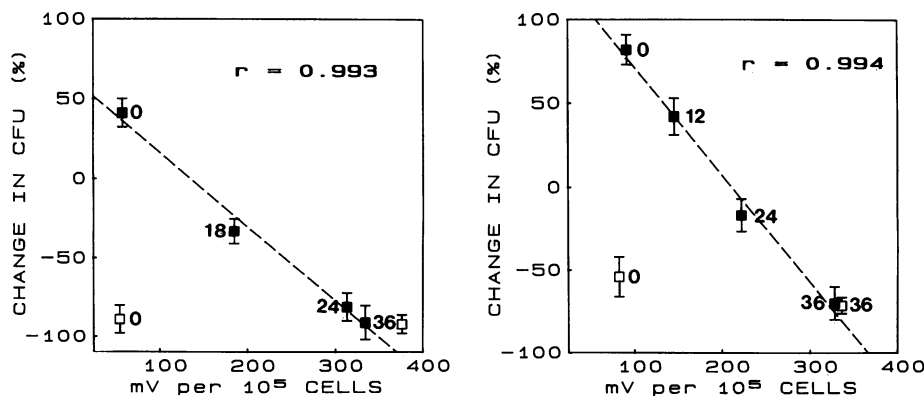


**Figure 6.** Effects of  $\gamma$ -interferon and dexamethasone on the antilisterial activity of blood-derived macrophages. Monocytes were exposed for 36 h to  $2.5 \times 10^{-7}$  M of dexamethasone (DEXA) or 0.001% ethanol (CONTROL) before challenge with *Listeria monocytogenes*. (IFN) Exposure of monolayers to 50 U/ml  $\gamma$ -interferon for 36, 24, and 18 h before challenge. Change in the percentage of CFU between baseline (after removal of noningested bacteria) and after incubation for 3 h. Mean  $\pm$ SEM from triplicate experiments with quadruplicate tubes for each cell preparation and group.



**Figure 7.** Relation of the change in CFU of bacteria determined by quantitative culture to the fate of intracellular bacteria followed visually by enumeration of the percentage of macrophages harboring bacteria. The tight relation between the change in the number of macrophages clearing bacteria in the presence of an aminoglycoside for suppression of extracellular growth (during the 8 h of incubation mandatory for digestion of bacteria) and the results obtained by quantitative cultures after incubation of infected, washed monolayers for only 3 h in antibiotic-free medium assures that the results from the method depending on quantitative culture also represent the fate of phagocytized bacteria. Pooled results from three independent experiments mean from parallel sets of quadruplicate wells and quadruplicate coverslips.

ROI, i.e., secretion of  $H_2O_2$  and  $O_2^-$ . These experiments confirmed that resting mononuclear phagocytes which secreted only a modest amount of  $H_2O_2$  had a high antilisterial activity which could barely be improved by activating systems responsible for secretion of ROI by  $\gamma$ -interferon (Fig. 8). Resting blood-derived macrophages exposed to dexamethasone lost their antilisterial activity independently from an impairment of the modest secretion of  $H_2O_2$  of resting phagocytes, which was identical to that of control cells (Fig. 8). Dexamethasone again did not disturb activation of systems responsible for secretion of  $H_2O_2$  (Fig. 8). However, in contrast to control monocytes which were already very active against *Listeria* in their "resting" state, activation of  $H_2O_2$ -secreting systems in dexamethasone-exposed cells by  $\gamma$ -interferon resulted reproducibly in a tight correlation between antilisterial activity and secretion of  $H_2O_2$  (Fig. 8). Comparable results were obtained in three additional independent experiments relating listericidal activity to secretion of  $O_2^-$  measured by lucigenin-amplified chemiluminescence (Fig. 9).



**Figure 8.** Effects of dexamethasone and  $\gamma$ -interferon on the relation of the change in CFU of *Listeria monocytogenes* phagocytized by human blood-derived macrophages to peak chemiluminescence in response to stimulation by PMA in a luminol-HRP-amplified system ( $H_2O_2$  release). Monolayers were exposed for various time spans before challenge with *Listeria* and parallel quantitation of chemiluminescence to 50 U/ml of  $\gamma$ -interferon. The numbers beside the symbols give the duration of lymphokine exposure before challenge. (□) Control cells; (■) cells exposed for

the whole duration of the experiment to  $2.5 \times 10^{-7}$  M of dexamethasone. Note that efficacious killing of control cells (left lower corner) can hardly be improved by  $\gamma$ -interferon exposure (□ right corner). After dexamethasone exposure, killing power and  $H_2O_2$  release become tightly related as shown by simple linear regression. Each panel gives the mean  $\pm$  SD from an independent experiment with quadruplicate wells.

Taken together these results suggested that resting human blood-derived macrophages efficiently killed *Listeria monocytogenes* by nonoxidative killing systems. Activation of alternate killing systems such as those responsible for secretion of ROI could therefore not substantially increase listericidal activity of normal monocytes not exposed to dexamethasone. In contrast, after elimination of nonoxidative antilisterial activity by dexamethasone, gradual activation of oxidative killing systems was paralleled by an according increment of the antilisterial activity, proportional to the amount of  $H_2O_2$  or  $O_2^-$  secreted in answer to  $\gamma$ -interferon. This concept was further substantiated by demonstrating that the antimicrobial activity of macrophages against *Aspergillus* spores, which is independent from oxidative killing systems (5, 9), could not be restored by  $\gamma$ -interferon in dexamethasone-treated cells despite of activated oxidative systems and reestablished antilisterial activity (Table I).

## Discussion

These studies on the interrelation of the effects of glucocorticoids and of the macrophage-activating lymphokine  $\gamma$ -interferon on macrophage function show that glucocorticoids impair antimicrobial activity of human blood monocytes without affecting secretion of antimicrobial oxygen metabolites by nonactivated, resting phagocytes and without preventing an enhancement of oxidative killing systems during activation by  $\gamma$ -interferon.

Glucocorticoids lower antimicrobial activity of macrophages, an immunosuppressive mechanism which appears to be critical for the effect of glucocorticoids on host resistance in several models of opportunistic infections such as listeriosis (3, 4), mycobacteriosis (1), and aspergillosis (2). We have previously reported that the deleterious effect of therapeutic glucocorticoid concentrations on macrophage function results from a direct, receptor-mediated steroid effect on the capacity of mononuclear phagocytes to kill ingested bacteria and fungal spores (2, 5). Based on observations that glucocorticoids impair antimicrobial activity of resting macrophages from patients with chronic granulomatous disease, which are unable to produce significant amounts of antimicrobial ROI, and from normal donors alike, we have proposed that glucocorticoids impair nonoxidative killing systems of "resting" macrophages (5). The present studies show that dexamethasone at pharma-

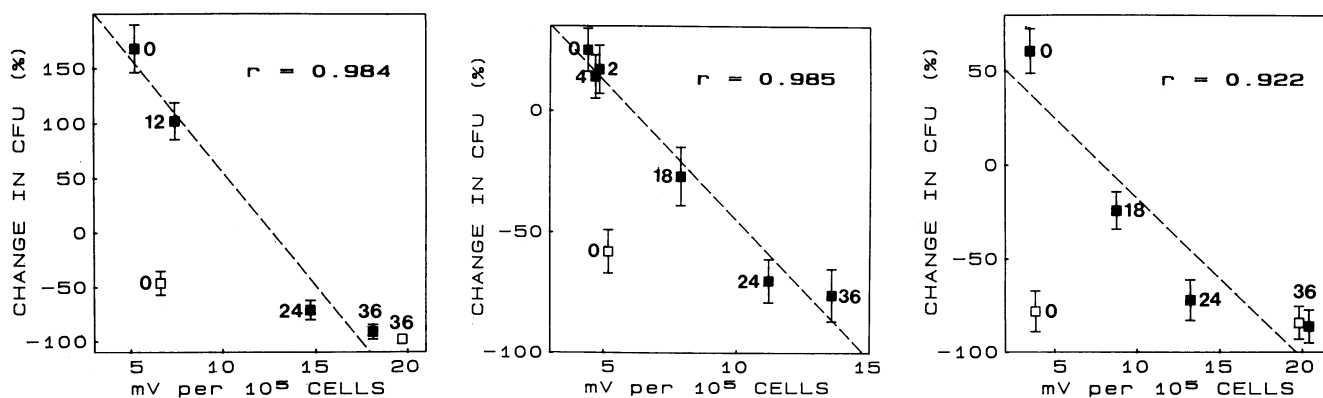


Figure 9. Effects of dexamethasone and  $\gamma$ -interferon on the relation of the change in CFU of *Listeria monocytogenes* phagocytized by human blood-derived macrophages to peak chemiluminescence in

response to stimulation by PMA in a lucigenin-amplified system ( $O_2^-$  release). For symbols and explanation see legend to Fig. 8. Each panel gives the mean  $\pm$  SD from an independent experiment.

colonic concentrations has no effect on the amount of ROI secreted by resting blood-derived macrophages upon stimulation with PMA, a soluble membrane perturber (Figs. 1 (upper panel), 2, 5, 8, and 9), opsonized particles (Figs. 1 (lower panel) 3), or live bacteria (Fig. 4). Dexamethasone does also not hinder activation of systems generating ROI by  $\gamma$ -interferon as shown by an identical activation of systems generating  $O_2^-$  and  $H_2O_2$  in dexamethasone-treated and control cells (Figs. 1–5, 8, and 9). Dexamethasone therefore impairs antimicrobial function by affecting nonoxidative killing systems, operative in the nonactivated state of resting mononuclear phagocytes.

Human blood monocytes kept in culture for 36 h, regardless of their limited capacity to produce ROI without activa-

tion, efficaciously kill ingested *Listeria* and do not require activation by lymphokines to exert this function (Figs. 6–9). Therefore activation by  $\gamma$ -interferon of alternate killing systems such as those generating ROI can only trivially improve the reliable activity of the nonoxidative killing systems of “resting” macrophages (Figs. 8 and 9). The importance of alternate, probably oxidative, killing systems is only brought out in this model after elimination of the dexamethasone-sensitive antilisterial activity. After a glucocorticoid-induced loss of antilisterial activity, gradual activation of systems generating ROI is paralleled by an accordingly increased activity against *Listeria* in dexamethasone-exposed cells (Figs. 8 and 9). That it is not merely a restoration of the function of the dexamethasone-sensitive, nonoxidative killing systems by  $\gamma$ -interferon can be shown by demonstrating that the defect in the antimicrobial activity against microorganisms which are more resistant against ROI such as *Aspergillus* spores (5, Table I) or *Nocardia asteroides* (5) persists in dexamethasone-exposed cells, despite of activated systems generating ROI. It appears therefore important to consider the susceptibility of a particular microorganism to the various killing systems of the phagocytic armature in studies of the functional modulation of antimicrobial activity of macrophages. For the same reasons it appears logical that glucocorticoids do not necessarily reduce activity of resting macrophages against all pathogens.

In conclusion, our studies indicate that dexamethasone impairs nonoxidative killing systems of resting macrophage which are operative against a broad spectrum of bacteria and fungi (e.g., *Listeria*, *Salmonella*, *Nocardia*, and *Aspergillus*).  $\gamma$ -Interferon, by activating alternate killing systems such as those generating antimicrobial ROI, enables macrophages to kill microorganisms sensitive to the antimicrobial armature operative in the activated state, even after elimination of dexamethasone-sensitive killing mechanisms.  $\gamma$ -Interferon does not restore activity of such dexamethasone-sensitive killing systems operative in resting macrophages.  $\gamma$ -Interferon therefore does not restore function against pathogens more resistant against antimicrobial systems of activated macrophages such as *Nocardia* (5) or *Aspergillus* spores (5, Table I), which are known to be relatively resistant to ROI.

Modulation of antimicrobial function of macrophages by the two opposing agents  $\gamma$ -interferon and dexamethasone permits distinguishing between contributions of various anti-

Table I. Comparison of the Effects of Dexamethasone and of  $\gamma$ -Interferon on the Activity of Macrophages against *Listeria* and *Aspergillus* Spores in Relation to  $H_2O_2$  Production

Exposure of macrophages	% spores inhibited*	% <i>Listeria</i> killed <sup>†</sup>	$H_2O_2$ production mV/10 <sup>5</sup> cells <sup>‡</sup>
Control	83 $\pm$ 6	71 $\pm$ 7	57 $\pm$ 18
Dexamethasone	11 $\pm$ 4	0	64 $\pm$ 13
$\gamma$ -Interferon	85 $\pm$ 7	82 $\pm$ 3	261 $\pm$ 35
Dexamethasone/ $\gamma$ -interferon	9 $\pm$ 6	86 $\pm$ 5	271 $\pm$ 41

Human blood monocytes were kept in culture for 36 h before challenge with *Aspergillus* spores or *Listeria* or analysis of  $H_2O_2$  secretion, and exposed for the duration of the experiment to  $2.5 \times 10^{-7}$  M dexamethasone, 50 U/ml of recombinant  $\gamma$ -interferon, a combination of dexamethasone and recombinant gamma interferon or solvent (control), which was 0.001% ethanol. Results are the mean value  $\pm$  SEM from triplicate experiments.

\* Activity against *Aspergillus* spores was quantitated by enumerating 24 h after phagocytosis the percentage of ingested spores prevented by macrophages from germination; control spores in cell-free medium had germinated by 18 h at a rate of > 95%.

<sup>†</sup> Percent reduction in CFU; in blood-derived macrophages treated with dexamethasone alone the number of CFU increased in each experiment over that of the baseline values.

<sup>‡</sup> Peak chemiluminescence in millivolts in the HRP-luminol-amplified system.

crobal systems of mononuclear phagocytes to host defense. The presented data on the effect of dexamethasone on nonoxidative killing systems of resting macrophages, which results in vivo in a loss of natural resistance against a broad range of microorganisms (1-5), lends to the hypothesis that nonoxidative killing systems of macrophages play a crucial role in natural immunity to bacteria and fungi. Indirect evidence points to oxidative killing systems taking an important role in the restoration of antimicrobial function by  $\gamma$ -interferon in glucocorticoid-treated monocytes.

### Acknowledgments

We thank Marlene Fischer for her expert technical help, and Biogen SA, Geneva, Switzerland for providing us with human recombinant  $\gamma$ -interferon.

This research was supported by grant 3.875.85 from the Swiss National Science Foundation.

### References

1. Lurie, M. B. 1964. Role of adrenal cortex in native resistance to tuberculosis. In *Resistance to Tuberculosis: Experimental Studies in Native and Acquired Defense Mechanisms*. M. B. Lurie, editor. Harvard University Press, Boston. 224-264.
2. Schaffner, A., H. Douglas, and A. Braude. 1982. Selective Protection against conidia by mononuclear and against mycelia by polymorphonuclear phagocytes in resistance to *Aspergillus*. *J. Clin. Invest.* 69:617-631.
3. Schaffner, A., H. Douglas, and C. E. Davis. 1983. Models of T cell deficiency in listeriosis: the effects of cortisone and cyclosporin A on normal and nude BALB/c mice. *J. Immunol.* 131:450-453.
4. Blackwood, L. L., and J. E. Pennington. 1982. Dose-dependent effect of glucocorticosteroids on pulmonary defense in a steroid-resistant host. *Am. Rev. Respir. Dis.* 126:1045-1049.
5. Schaffner, A. 1985. Therapeutic concentrations of glucocorticoids suppress the antimicrobial activity of human macrophages without impairing their responsiveness to gamma interferon. *J. Clin. Invest.* 76:1755-1764.
6. Nathan, C. F., H. W. Murray, M. E. Weibe, and B. Y. Rubin. 1983. Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J. Exp. Med.* 158:670-689.
7. Schultz, R. M., and W. J. Kleinschmidt. 1983. Functional identity between murine gamma-interferon and macrophage activating factor. *Nature (Lond.)*. 305:239-240.
8. Nagakawara, A., C. F. Nathan, and Z. A. Cohn. 1981. Hydrogen peroxide metabolism in human monocytes during differentiation in vitro. *J. Clin. Invest.* 68:1243-1252.
9. Schaffner, A., H. Douglas, A. I. Braude, and C. E. Davis. 1983. Killing of *Aspergillus* spores depends on the anatomical source of the macrophage. *Infect. Immun.* 42:1109-1115.
10. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
11. Root, R. K., J. Metcalf, N. Oshino, and B. Chance. 1975. H<sub>2</sub>O<sub>2</sub> release from human granulocytes during phagocytosis. I. Documentation, quantitation, and some regulating factors. *J. Clin. Invest.* 55:945-955.
12. Allen, R. C., and L. D. Loose. 1976. Phagocytic activation of a luminol-dependent chemiluminescence in rabbit alveolar and peritoneal macrophages. *Biochem. Biophys. Res. Commun.* 69:245-252.
13. Schaffner, A., C. E. Davis, T. Schaffner, M. Markert, H. Douglas, and A. I. Braude. 1986. In vitro susceptibility of fungi to killing by neutrophil granulocytes discriminates between primary pathogenicity and opportunism. *J. Clin. Invest.* 78:511-524.
14. Müller-Peddinghaus, R. 1984. In vitro determination of phagocyte activity by luminol- and lucigenin-amplified chemiluminescence. *Int. J. Immunopharmacol.* 6:455-466.
15. Allen, R. C. 1981. Lucigenin chemiluminescence: a new approach to the study of polymorphonuclear redox activity. In *Bioluminescence and Chemiluminescence*. M. A. DeLuca and W. D. McEbroy, editors. Academic Press, Inc., New York. 63-73.
16. Sachs, L. 1978. *Angewandte Statistik*. 6th edition. Springer-Verlag, Berlin. 1-552.