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# EFFECTS OF ANTI-INFLAMMATORY AND IMMUNO-MODULATING AGENTS ON THE RELEASE OF β-GLUCURONIDASE AND COLLAGENASE FROM CULTURED MACROPHAGES OF GUINEA PIGS

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**Abstract**—The effects of various drugs on zymosan-induced release of β-glucuronidase (a lysosomal enzyme) and lipopolysaccharide (LPS)-induced secretion of collagenase from cultured macrophages of guinea pigs were investigated. Dexamethasone, hydrocortisone and prednisolone inhibited the release of β-glucuronidase. Dexamethasone, however, did not show such an effect, when the drug was added to macrophage culture after particle uptake had completed. In contrast, indomethacin and disodium *N*-(carboxyphenyl)4-chloroanthranilate (CCA) significantly enhanced the enzymic release. Aspirin and levamisole enhanced it slightly. But there were no distinct differences between the effects of these agents on LPS-induced secretion of collagenase in contrast to the case of β-glucuronidase release.

It has been shown that, during phagocytosis, macrophages release lysosomal enzymes and neutral proteases like collagenase which might be responsible for tissue damages at the site of chronic inflammation [1]. The significance and mechanism of this phenomenon have not, however, been clearly elucidated. It has been reported that glucocorticoids inhibited lysosomal enzyme release from macrophages stimulated by zymosan [2]. Nonsteroidal anti-inflammatory agents, however, showed no inhibition. This may correlate well with the effectiveness of glucocorticoids against chronic inflammation.

From the immunological point of view, glucocorticoids are typical immunosuppressive agents which eliminate immature T-cells or suppress accessory cell (A-cell) activity of macrophages [3, 4]. On the other hand, various drugs like levamisole and CCA have recently been tried to treat chronic inflammation and autoimmune disease [5]. These agents are predicted to express their therapeutic effects through actions on immune system.

The present paper describes the effects of various drugs including anti-inflammatory and immuno-modulating agents on the release of lysosomal enzymes and collagenase from guinea pig macrophages. A correlation between the effects on these systems and immuno-modulating properties is discussed.

## MATERIALS AND METHODS

*Materials.* Medium 199 and Dulbecco's modified Eagle's medium (DMEM) were purchased from Nissui Seiyaku Co., Ltd. Foetal calf serum was the product of GIBCO. *p*-Nitrophenyl-β-D-glucuronide, zymosan and hydrocortisone were obtained from Sigma Chemical Co., Ltd. Synthetic peptide (DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH) was obtained from peptide Kenkyusho Co., Ltd. and LPS derived from *E. coli* (055;B5) was purchased from Difco Laboratories. Drugs used in the investigation were kindly given as follows; indomethacin, phenylbutazone, aspirin, flufenamic acid, dexamethasone and prednisolone by Santen Seiyaku Co., Ltd.; levamisole, penicillamine and gold salt by Teijin Institute for Biomedical Research; CCA by Chugai Seiyaku Co., Ltd. All other chemicals used were of the highest purity available.

### *Effects of drugs on β-glucuronidase release*

*Macrophage culture.* Macrophages were obtained from the peritoneal cavity of guinea pigs 4 days after the injection of 10 ml of sterile liquid paraffin. Medium 199 (1.5 ml) containing $6 \times 10^6$ washed cells per ml was placed in tissue culture dishes ($35 \times 10$ mm style; Falcon) and incubated in an atmosphere of 5% $CO_2$ and 95% air at 37° according to the method of Stuart *et al.* with some modifications [6]. After incubation for 12–15 hr, the nonadherent cells were removed by several washings with prewarmed saline, and pre-incubated in 1.5 ml of fresh Medium 199 containing drugs. After 1 hr incubation, zymosan was added to give $2 \times 10^7$ particles/ml and incubation continued. After 6 hr, the culture medium was recovered, centrifuged and aliquots of the supernatants were assayed for β-glucuronidase and lactate dehydrogenase (LDH) activities, in order to estimate the specific release of enzymes from cultured cells.

*Preparation of zymosan particles.* Zymosan suspension (10 mg/ml, i.e. $5 \times 10^8$ particles/ml) were boiled and washed according to the method of Weissmann *et al.* [7].

*Drug solution.* All compounds, which were not water soluble, were dissolved in demethyl sulphoxide

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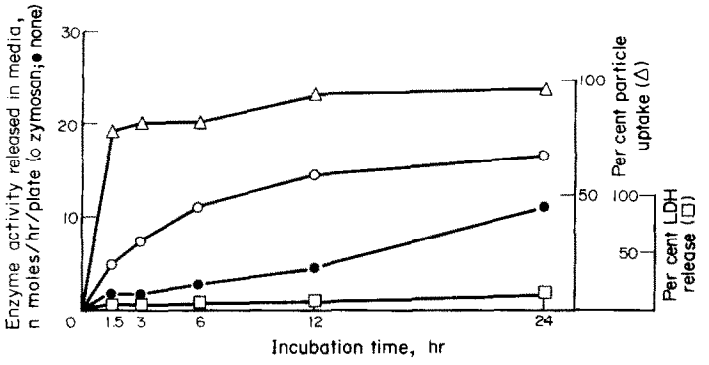


Fig. 1. Effect of zymosan particles on the release of β-glucuronidase and lactate dehydrogenase (LDH) from cultured macrophages. Macrophages ($6 \times 10^6$ cells) were incubated in the presence or absence of zymosan ($3 \times 10^7$ particles). At the indicated time, the supernatants were collected and assayed for enzyme activities as described in Materials and Methods. LDH release was expressed as a percentage of the total enzyme activity of the culture which was estimated after six cycles of freezing and thawing. Particle uptake was expressed as per cent ingested cells of 100 cells observed.

(DMSO) and added to the culture medium to give the final DMSO concentration of 0.1% (v/v). DMSO at 0.1% (v/v) did not affect enzyme release and cell viability.

*Enzyme assays.* LDH and β-glucuronidase activities were determined by some modifications of the procedures of Kornberg and Fishman, respectively [8, 9].

*Measurement of phagocytosis.* Uptake of zymosan particles by macrophages was quantitated by enumeration of ingested particles by light microscopy.

### *Effects of drugs on collagenase secretion*

Macrophages ($6 \times 10^6$ cells) were cultured with 30 μg/ml of LPS in 1.5 ml DMEM under 5% $CO_2$ and 95% air at 37° [10]. The medium was changed every 24 hr. In some cases, 10 μl of various drugs dissolved in 15% DMSO were added to the culture. Collagenase activity in the medium from 48 hr culture was assayed by using synthetic peptide (DNP-Pro-Gln-Ile-Ala-Gly-Gln-D-Arg-OH) as reported by Nagai *et al.* [11].

## RESULTS

*Effect of zymosan particles on enzyme release from cultured macrophages.* Figure 1 shows that after zymosan was added to macrophage culture, the release of β-glucuronidase was initiated and continued during the 24 hr of incubation. But, in contrast, particle uptake by macrophage was very rapid and reached 80 per cent of maximum uptake within 1.5 hr. There was no significant release of lactate dehydrogenase (LDH; a cytoplasmic enzyme) during 24 hr of incubation, thus indicating that nonspecific lysis of cells did not occur. Figure 2 shows the effects of zymosan on β-glucuronidase activity in the total culture, in the cells and in the culture medium. The level of β-glucuronidase in total cultures remained constant over a period of 24 hr. Thus, it is considered that the enzyme was released from the intracellular pool, and was not synthesized *de novo*. This is consistent with the observation in the case of β-glucuronidase release from mouse macrophages stimulated by peptidoglycan [12].

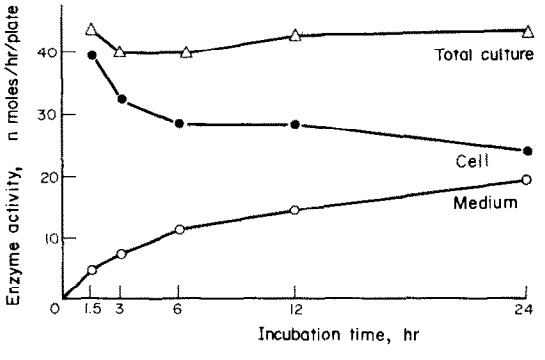


Fig. 2. Effect of zymosan on β-glucuronidase activity in the total culture, in the cells and in the culture medium at time intervals over a period of 24 hr. Macrophages ($6 \times 10^6$ cells) were incubated with zymosan particles ($3 \times 10^7$ particles) as described in Materials and Methods. Intracellular enzyme activities were determined after six cycles of freezing and thawing. Total enzyme activities were expressed as the sum of activities in the culture medium and in the cells.

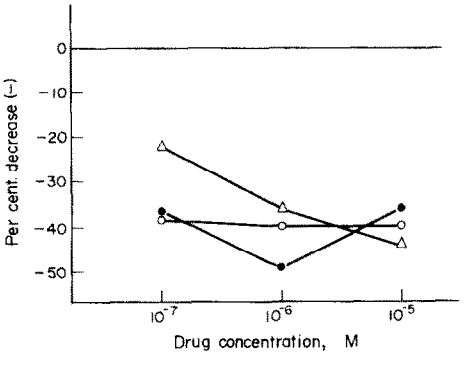


Fig. 3. Effect of glucocorticoids on zymosan-induced release of β-glucuronidase from cultured macrophages. Macrophage cultures ($6 \times 10^6$ cells) were preincubated for 1 hr with dexamethasone (○), prednisolone (●) and hydrocortisone (△) at the molarities indicated. Zymosan ($3 \times 10^7$ particles) was added and incubation continued for 6 hr. β-Glucuronidase activities were determined in the culture supernatants. The data were expressed as a per cent decrease (minus value) from the control release.

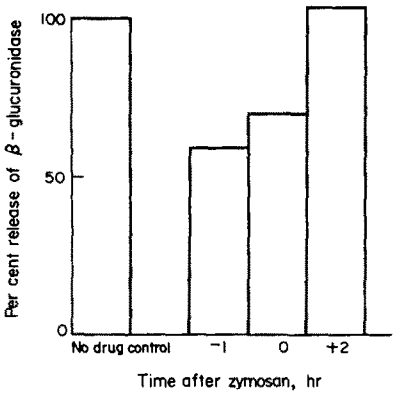


Fig. 4. Zymosan-induced release of β-glucuronidase from cultured macrophages received dexamethasone at different times. Macrophages ($6 \times 10^6$ cells) were incubated with zymosan ($3 \times 10^7$ particles) as described in Materials and Methods. Dexamethasone ($2 \times 10^{-5}$ M) was added at the indicated time before or after the addition of zymosan. β-Glucuronidase activities were determined in the culture supernatants 6 hr after zymosan.

*Effect of glucocorticoids on zymosan-induced release of β-glucuronidase from cultured macrophages.* The results in Fig. 3 indicate that dexamethasone, prednisolone and hydrocortisone significantly inhibited zymosan-induced release of β-glucuronidase from macrophages. The inhibitory effects were still observed at $10^{-7}$ M of these drugs, but did not exceed 50 per cent at higher drug concentrations.

*Zymosan-induced release of β-glucuronidase from cultured macrophages received dexamethasone at different times during incubation.* The results stated above indicated the inhibitory effects of glucocorticoids on the enzyme release from macrophages. On the other hand, it was observed that dexamethasone inhibited about 20–30 per cent of the uptake of zymosan by these cells. Figure 4 shows that this inhibition of the enzyme release by dexamethasone was observed only when the drug was added to macrophage culture 1 hr before or simultaneously with the addition of zymosan. Therefore, it appears that this inhibition of the enzyme release would be due to the suppression of particle uptake of zymosan.

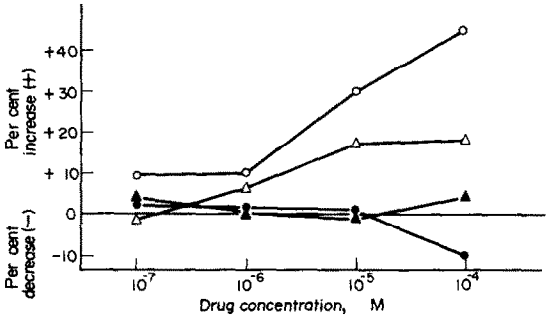


Fig. 5. Effects of nonsteroidal anti-inflammatory agents on zymosan-induced release of β-glucuronidase from cultured macrophages. Macrophage cultures ($6 \times 10^6$ cells) were preincubated for 1 hr with indomethacin (○), flufenamic acid (●), aspirin (△) and phenylbutazone (▲) at the molarities indicated. Zymosan ($3 \times 10^7$ particles) was added and incubation continued for 6 hr. β-Glucuronidase activities were determined in the culture supernatants. The data were expressed as a percent increase (+) or decrease (−) from the control release.

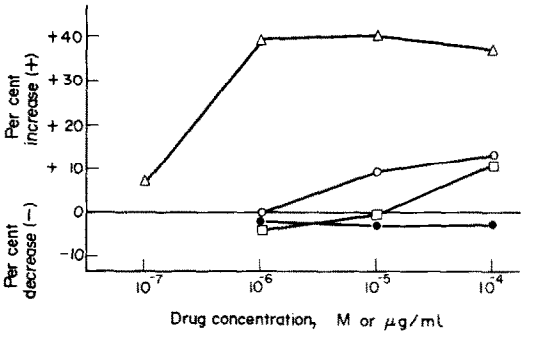


Fig. 6. Effects of levamisole, gold salt (thiomalate) and CCA on zymosan-induced release of β-glucuronidase from cultured macrophages. Macrophage cultures ($6 \times 10^6$ cells) were preincubated for 1 hr with levamisole (□), penicillamine (●), gold salt (○) (μg/ml) and CCA (△) at the molarities indicated. Zymosan ($3 \times 10^7$ particles) was added and incubation continued for 6 hr. β-glucuronidase activities were determined in the culture supernatants. The data were expressed as a per cent increase (+) or decrease (−) from the control release.

*Effects of nonsteroidal anti-inflammatory agents on zymosan-induced release of β-glucuronidase from cultured macrophages.* At $10^{-6}$–$10^{-7}$ M, indomethacin, flufenamic acid, aspirin and phenylbutazone had no effect on the release of β-glucuronidase from macrophages. It is interesting to note, however, that indomethacin and aspirin, at $10^{-4}$–$10^{-5}$ M, enhanced the enzyme release significantly, as shown in Fig. 5. LDH activity was not detected in the culture medium in these cases. This indicates that the enhancement of the enzyme release would not be due to the lysis of macrophages.

*Effects of levamisole, penicillamine, gold salt (thiomalate) or CCA on zymosan-induced release of β-glucuronidase from cultured macrophages.* it was then investigated whether immuno-modulating agents or drugs applied for the treatment of chronic inflammation would have some effects on zymosan-induced release of β-glucuronidase from cultured macrophages. Penicillamine had no effect on the release of β-glucuronidase as shown in Fig. 6. But, CCA, which is known to be effective against rheumatoid arthritis [5] and potentiate humoral immune response [13], significantly enhanced zymosan-induced release of the enzyme. A slight enhancement was also observed with levamisole, another immuno-modulating agent, which is known to potentiate cell-mediated and humoral immune responses [14–16]. CCA and levamisole did not release lactate dehydrogenase from macrophages.

*Effects of steroidal and nonsteroidal anti-inflammatory agents on the secretion of collagenase activity from guinea pig macrophages.* It has been reported that macrophages when cultured with LPS secrete collagenase into medium after 24 hr of lag time [10]. This phenomenon is considered to be a marker of macrophage activation. The enzyme secretion is known to be suppressed by indomethacin [10]. We investigated whether various anti-inflammatory agents can affect this collagenase secretion. Figure 7 indicates that both steroidal

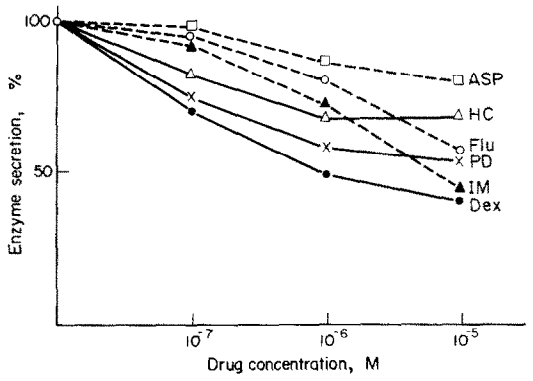


Fig. 7. Effects of steroidal or nonsteroidal anti-inflammatory agents on the secretion of collagenase activity from guinea pig macrophages. Macrophages ($6 \times 10^6$ cells) were incubated with LPS (30 μg/ml) for 48 hr. The indicated concentrations of various drugs were added at the start of the culture. Supernatants from day 2 cultures were assayed for the enzyme activity. Drugs added were aspirin (□), hydrocortisone (△), flufenamic acid (○), prednisolone (×), indomethacin (▲) and dexamethasone (●).

and non-steroidal anti-inflammatory agents slightly suppressed the enzyme secretion, but the former group (especially dexamethasone) showed relatively more potent effect. There were no distinct differences between the effects of steroidal and non-steroidal anti-inflammatory agents in contrast to the case of lysosomal enzyme release.

## DISCUSSION

The results indicate that glucocorticoids suppressed zymosan-induced lysosomal enzyme release from macrophages. It has been demonstrated that drugs such as hydrocortisone inhibited the release of hydrolases from isolated lysosomes by stabilizing lysosomal membranes [17, 18]. Smith reported that glucocorticoids inhibited phagocytosis in guinea pig neutrophils, thus leading to the inhibition of lysosomal enzyme release [19]. In the present experiment, we observed that dexamethasone had no effect when the drug was added to macrophage culture after particle uptake had completed, indicating that dexamethasone reduced the enzyme release by inhibiting the uptake of zymosan particles. If stabilization of lysosomal membranes would be the major factor in the effects of the drug, the inhibition of the enzyme release should be observed independently of the time of the drug addition. Thus, the inhibition of the enzyme release by glucocorticoids may be due to the suppression of phagocytosis. This inhibitory effects of the enzyme release by glucucorticoids did not exceed about 50 per cent. Recently, it is reported that there are some subpopulations in macrophages with different functions in immune responses [20]. Then, it would be suggested that this limited inhibitory effect may be due to heterogeneity of macrophages, i.e. there may exist subpopulations in macrophages with different sensitivities to glucocorticoids.

In contrast to glucocorticoids, other drugs such as CCA, levamisole and indomethacin enhanced zymosan-induced release of β-glucuronidase from macrophages. These agents have been reported to enhance various immune responses. CCA increased the production of splenic hemolytic plaque-forming cells (PFC) against both thymus-dependent and thymus-independent antigens in mice [13]. Levamisole increased the production of PFC against sheep red blood cells in mice [16]. Indomethacin enhanced mitogen-induced stimulation of human lymphocyte [21]. On the other hand, glucocorticoids are reported to suppress immune responses. It is interesting to note that there is some correlation between the effect on zymosan-induced release of β-glucuronidase from macrophages and those on immune responses. Macrophage is known to play a vital role as A-cell in the humoral immune response or the induction of cytotoxic T-cell.

Antigenic substances are reported to activate macrophages. There are various criteria as for the activation of macrophages; the lysosomal enzyme release, the secretion of collagenase or plasminogen activator, the phagocytosis, morphological changes etc. [22, 23]. Our results suggest a correlation between activation of A-cell function and that of macrophage (*i.e.* the lysosomal enzyme release).

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