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DEXAMETHASONE INHIBITS ANTITUMOR POTENTIAL OF ACTIVATED MACROPHAGES BY A RECEPTOR MEDIATED ACTION

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The capacity to inhibit P815 mastocytoma growth was induced in macrophages elicited by trehalose dimycolate by a short in vitro treatment with 10 ng/ml LPS. Activation by LPS was associated with a 3 fold increase in the rate of glucose consumption by macrophages. Incubation of activated macrophages with the glucocorticoid dexamethasone ($\geq 10^{-8}$M) for several hours ($\geq$ 5 h) resulted in an inhibition of antitumoral activity and a decrease of glucose consumption. Hydrogen peroxide production is a property expressed by trehalose dimycolate-elicited macrophages independently of the presence of LPS. The capacity to release hydrogen peroxide upon triggering was not affected by a pretreatment of macrophages by dexamethasone.
The antiglucocorticoid compound RU 38486, known to bind with a high affinity to glucocorticoid receptors without agonist effect, prevented the inhibitory actions of dexamethasone, indicating that these are receptor-mediated.

Mouse peritoneal macrophages, when appropriately activated, inhibit tumor cell growth during coculture in vitro and destroy susceptible tumor targets by a non-phagocytic process (1). The precise mechanism whereby macrophages or their products attack target cells is poorly understood. Possible effector molecules include arginase (2), neutral proteases (3), cytotoxic protein factors such as Tumor Necrosis Factor (4), toxic oxygen species (5), a metal chelator (6) and lipoxygenase derivatives (7). In an attempt to recognize effector mechanisms responsible for macrophage antitumor action, we examined the effects on activated macrophages of dexamethasone, a drug which inhibits the expression of cytotoxicity in various models (8, 9, 10, 11).

Corticosteroids have been shown to modify a variety of responses in different macrophage populations and macrophage cell lines, especially the production of proteins which are not constitutively synthesized, such as plasminogen activator (12) or other neutral proteases (13, 14), Colony Stimulating Factor (15) and Interleukin I (16). We thus hypothesized that

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dexamethasone might inhibit the production of an hypothetical proteinic cytotoxin.

Furthermore, in various experimental systems, including macrophages, corticosteroids prevent arachidonic release from phospholipids by inducing a phospholipase A_2 inhibitory protein (17, 18, 19). A blockade of the release of arachidonic derivatives might also interfere with the antitumor action of macrophages if some of these derivatives act directly on tumor cells (7) or trigger the release of toxic oxygen species (20).

In fact, we observed that activated macrophages treated by dexamethasone lost their capacity to inhibit tumor cell growth and we explored which functions of activated macrophages were inhibited simultaneously with antitumor action. By using the antiglucocorticoid RU 38486 which binds with a high affinity to glucocorticoid receptors (21, 22, 23), we demonstrated that the inhibitory potential of dexamethasone on activated macrophages is truly receptor-mediated.

MATERIALS AND METHODS

Reagents Trehalose dimycolate (TDM), prepared from Mycobacterium tuberculosis, strain Peurois, was obtained from Choay Chimie (France) and suspended in water according to the method of Kato (24). Fetal calf serum (FCS) was obtained from Gibco (Great Britain). FCS and reagents were screened for endotoxin by the Limulus amoebocyte lysate assay as previously described (25). FCS containing < 1 ng/ml endotoxin was used. LPS (extracted from Salmonella enteritidis by the Westphal method) was purchased from Difco; [^{3}H]thymidine (29 Ci/mmole) was from CEA (France), TPA from Sigma and zymosan from ICN Pharmaceuticals. Dexamethasone and RU 38486 were generous gifts from Roussel Uclaf (France).
Macrophages Peritoneal macrophages were collected from female (C57Bl/6 x DBA/2) F1 mice purchased from Charles River (France); animals received 50 µg of TDM i.p. 7 days before the harvest. Peritoneal cells (1.2 x 10^6 macrophages/ml) in MEM, supplemented with antibiotics and 5% heat-inactivated FCS, were plated in microtest plates (250 µl/well); LPS (10 ng/ml) was added, except in control wells. After 4 h at 37°C, non-adherent cells were washed out and macrophage monolayers were treated with steroids. Macrophages incubated in the absence of LPS were devoid of cytostatic activity: they will be referred to as primed macrophages. Macrophages incubated in the presence of LPS were fully activated to cytotoxicity.
Assays The cytostatic effect of macrophages on P815 mastocytoma cells was quantified by a [^{3}H]thymidine cumulative incorporation assay (26, 27). The release of H_2O_2 was measured from the horseradish peroxidase mediated oxidation of phenol red as proposed by Pick and Keisari (28) with slight modifications (25). The glucose concentration in culture media was measured as previously described (25) using the kit commercialized by Biotrol (France).

RESULTS AND DISCUSSION

The activation of macrophages for cytotoxicity against tumor cells is a stepwise process. In recent work, we have reported that mouse peritoneal macro-

phages elicited *in vivo* by trehalose dimycolate have the capacity to release large quantities of hydrogen peroxide upon triggering but are devoid of anti-tumoral activity against P815 mastocytoma cells (25). To become able to limit P815 growth, macrophages, primed by TDM action *in vivo*, need to receive a second activating signal, delivered by exposure to LPS during the first hours of culture *in vitro* (25). When applied to TDM-primed macrophages low doses of LPS (10 ng/ml) induce the expression of cytostatic activity and evoke a strong increase in the rate of glucose consumption ; the same doses of LPS are inefficient on macrophages which are not in the primed state.

We examined the effects of dexamethasone on three parameters - capacity to produce hydrogen peroxide, glucose consumption and cytostatic activity against tumor cells - which are acquired by macrophages along the activation sequence.

As shown in Table 1, exposure of activated macrophages to dexamethasone before the addition of tumor cells suppressed their antitumor activity. To observe an inhibition of macrophage cytostatic activity, the conditions of treatment by dexamethasone have to be selected carefully; 1) short exposures

TABLE 1: Effect of dexamethasone on expression of cytostasis in activated- macrophages

Dexamethasone		
during pretreatment of MØ	during cytostasis assay	Percentage growth inhibition (mean ± SD of n experiments)
0	0	88 % (± 5 %) n = 4
10^{-8} M	10^{-8} M	48 % (± 19 %) n = 3 ($p < 0.02$)
10^{-7} M	10^{-8} M	8 % (± 7 %) n = 4 ($p < 0.001$)

Macrophages (MØ)(1.2×10^6 cells/ml), after adherence (4 h) in a medium containing LPS, were incubated 9 h with dexamethasone 0, 10^{-8}M or 10^{-7}M, washed and added with P815 (0.3×10^6 cells/ml), [^{3}H]thymidine and dexamethasone 0 or 10^{-8}M. Inhibition of P815 growth was calculated according to the formula:

$$\% \text{ GI} = 100 \left[1 - \frac{\text{P815 growth in the presence of activated macrophages}}{\text{P815 growth in the presence of primed macrophages}} \right]$$

[^{3}H]Thymidine incorporated by P815 in control wells was 30 000 dpm.
Statistical analysis of the effect of dexamethasone was carried out using Student's two-tailed t test.

(of less than 4 h) are inefficient. 2) since macrophage cytostatic activity is a transient phenomenon, treatments longer than 9 h cannot be performed. 3) dexamethasone per se had an inhibitory effect on P815 growth (inhibited by 26% or 12% after 12 h in the presence of 10^{-7}M or 10^{-8}M dexamethasone respectively). Thus, activated macrophages were pretreated by 10^{-7}M or 10^{-8}M dexamethasone for 9 h, then their cytostatic power was measured in a 12 h assay, conducted in the presence of 10^{-8}M dexamethasone. Under such conditions, dexamethasone completely suppressed the antitumor activity induced by LPS.

Treatment of activated macrophages by dexamethasone also reduced their rate of glucose consumption : in activated macrophages treated by dexamethasone the rate of glucose consumption returned to a base level equal to that of resident or primed macrophages (Table 2).

In contrast, dexamethasone had no effect on the capacity to release high quantities of hydrogen peroxide upon triggering. The quantity of H_2O_2 released after 90 min by activated macrophages treated by dexamethasone was slightly higher than with control macrophages (Table 3). Similar results were obtained with two different triggering agents, TPA (tetradecanoyl phorbol acetate) and zymosan. These data thus differ from the findings of Schultz et al., who observed a 50% inhibition of response to TPA in macrophages treated for 18 h by dexamethasone (9). In TDM-elicited macrophages, the capacity to release large quantities of H_2O_2 upon triggering is not dependent upon LPS (25).

TABLE 2: Effet of dexamethasone on the rate of glucose consumption by activated macrophages

Macrophage state	Dexamethasone	Rate of glucose consomption µg of Glucose / h / 10^6 macrophages (mean ± SD of n experiments)		
Primed macrophages	0	4,4 (± 0,8)	n = 5	
Activated macrophages	0	12,0 (± 2,7)	n = 7	
	10^{-7}M	4,5 (± 0,5)	n = 2	($p < 0.01$)
	10^{-6}M	3,9 (± 0,2)	n = 2	($p < 0.01$)

After adherence (4 h) without LPS (primed macrophages) or with LPS (activated macrophages), macrophages were incubated 8 h with dexamethasone, 0, 10^{-7}M and 10^{-6}M. Glucose concentration in the culture media was measured on aliquots withdrawn at various time intervals.

TABLE 3: Dexamethasone failed to inhibit hydrogen peroxide production by activated macrophages

Macrophage state	Dexamethasone	Triggering agent	H_2O_2 production (nanomoles of H_2O_2/ 10^6 MØ /90min)	
Primed MØ	0	0	< 2	
		TPA	35 (± 9)	n = 2
		Zymosan	27 (± 2)	n = 2
Activated MØ	0	0	< 2	
	0	TPA	32 (± 9)	n = 6
	10^{-7} M	TPA	41 (± 4)	n = 2
	10^{-6} M	TPA	40	
	0	Zymosan	25 (± 5)	n = 5
	10^{-7} M	Zymosan	28 (± 5)	n = 2
	10^{-6} M	Zymosan	27 (± 3)	n = 2

After adherence (4 h) with or without LPS, macrophages (MØ)(1×10^6/ml) were treated with dexamethasone for 8 h , washed and incubated in the presence of TPA (10^{-7}M) or zymosan (400 µg/ml) as triggering agents.

The failure of dexamethasone to inhibit such a parameter may be related to the observation of Ralph et al., who found that corticosteroids had no effect on the constitutive functions of macrophage cell lines but inhibited the properties induced by LPS (15).

The capacity of the antiglucocorticoid agent RU 38486 to prevent the effects of dexamethasone is illustrated in Fig. 1. When RU 38486 was added

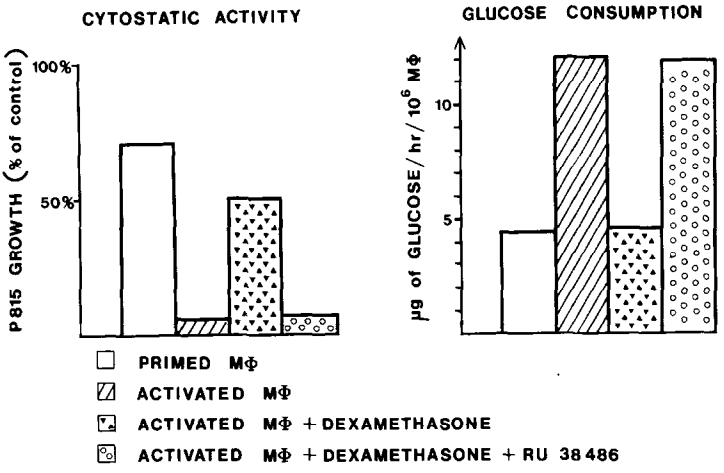

Figure 1: RU 38486 prevented the action of dexamethasone on activated macrophages.
After adherence (4 h) macrophages (MØ) were incubated 5 h in the presence of RU 38486 10^{-5}M, dexamethasone 10^{-6}M, or RU 38486 10^{-5}M + dexamethasone 10^{-6}M. After this treatment, macrophage monolayers were washed 3 times and reincubated in fresh medium for the assays of cytostatic activity and glucose consumption. RU 38486 (10^{-5}M) alone had no effect on macrophage cytostatic activity or glucose consumption.

15 min before dexamethasone, in a 10 fold excess, the inhibitory effects of dexamethasone on cytostatic activity and glucose consumption of activated macrophages were abolished. RU 38486 alone had no effect on these two parameters. RU 38486 represents a new class of 11β substituted steroid antihormones, without agonist effects (23); it has been demonstrated that RU 38486 binds with a high affinity to glucocorticoid receptors and that the binding is followed by an impaired activation (22). Thus, since RU 38486 prevented the effects of dexamethasone on activated macrophages, we concluded that the reversion of the biochemical changes seen in the activated state of macrophages after dexamethasone treatment is classically mediated by the binding of the drug to its receptor. We have now to investigate whether the suppressive action of dexamethasone observed here is due to the synthesis of inhibitor(s) and in particular whether the well-characterized one, lipocortine, is implicated.

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