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INDUCTION OF INTERFERON SYNTHESIS AND CYTOTOXICITY BY MURINE PERITONEAL MACROPHAGES EXPOSED TO GLYCOPROTEIN LIGANDS

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Summary. - Thioglycollate-induced murine C57BL/6 and C3H/HeN peritoneal macrophages synthesized interferon-beta (IFN-beta) in response to exposure to glycoproteins such as horseradish peroxidase (HRP) or mannosyl or fucosyl bovine serum albumin (BSAman of BSAfuc, respectively), but not glucosylated or galactosylated BSA (BSAglu or BSAgal, respectively). These results suggest participation of the mannosyl-fucosyl receptor (MFR) in this response. IFN synthesis was augmented by culturing macrophages in L cell-conditioned medium prior to exposure to these substances. Macrophages obtained from lipopolysaccharide (LPS)-resistant C3H/HeJ mice did not produce IFN in response to HRP. Furthermore, IFN-induction by HRP was blocked by polymyxin B. In addition, exposure of macrophages to HRP or BSAman induced cytotoxicity against NIH 3T12 cells. Cytotoxicity was not inhibited by the presence of anti-IFN-alpha/beta. In contrast to IFN induction, however, macrophages activation was LPS-independent, since this activity was demonstrated in macrophages from C3H/Hej mice. The carbohydrate specificity of these responses suggests that the MFR or an another scavenger receptor may be involved in the responses to these substances, and that cytotoxicity and IFN-induction by glycoproteins follow unique pathways.

Key words: macrophages; interferon; glycoproteins; peroxidases

Introduction

Macrophages play a key role in the generation of immune responses, and are thought to be involved in defense against neoplasia (Old *et al.*, 1961; Cohn,

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1978). In addition, macrophages are a major source of immunoregulatory compounds such as IFN and tumour necrosis factor (Werb and Goldstein, 1987). Many substances have been shown to regulate macrophages functions *in vitro,* including lymphokines, bacterial products and viral components (Adams and Marino, 1984; Hibbs, 1984; DeMaeyer and DeMaeyer-Guignard, 1988). IFN-induction by these compounds *in vivo* might be important to the outcome of viral disease or neoplastic transformation.

Peroxidases are naturally occurring enzymes which are found in the granules of polymorphonuclear neutrophils and eosinophils. These enzymes are released during degranulation and have been shown to be endocytosed by macrophages (Heifets *et al.*, 1980; Shellito *et al.*, 1987). Wei *et al.* (1986) described the cytotoxic activation of macrophages following exposure to peroxidative enzymes including HRP and bovine lactoperoxidase. Subsequently, Lefkowitz *et al.* (1989) demonstrated that peroxidase-induced macrophage-mediated cytotoxicity was dependent on the production of tumour necrosis factor.

The present study shows that exposure of macrophages to peroxidases or certain BSA glycoconjugates results in IFN synthesis in addition to the activation of macrophages to the cytotoxic state. The ability of these glycoproteins to stimulate macrophages appears to be dependent on the presence of mannose or fucose groups. These findings suggest a possible role for peroxidases and other glycoproteins as regulators of macrophage function.

Materials and Methods

Mice. 8–12 weeks old male and female C57BL/6, C3H/Hej, and C3H/HeN mice were obtained from Sasco.

Reagents. HRP Type VI, LPS (*E. coli* 127:B8), glycosylated BSA, polymyxin B, and HEPES were obtained from Sigma Chemical Co. Peroxidases and glycosylated BSA were filter-sterilized prior to use. Poly I:C was purchased from Boehringer-Mannheim Biochemicals. Antisera against murine IFN-alpha/beta and IFN-beta were purchased from Lee Biomolecular Laboratories. All reagents were analyzed for LPS contamination by Limulus assay (Associates of Cape Cod). Peroxidase were found to contain $\leq$0.38 ng of LPS/100 μg of protein.

Dulbecco's minimal essential medium (DMEM) was obtained from Gibco. Foetal bovine serum (FBS) was purchased from Sterile Systems.

Macrophages. Thioglycollate-induced macrophages were harvested 96 hrs after i. p. injection of 1 ml of Brewers Thioglycollate (Becton-Dickinson). Erythrocytes were lysed by the addition of 0.83 % NH_4Cl. The cells were cultured in 96 well dishes at $1x10^5$ cells/well. DMEM supplemented with 5 % FBS and 25 mmol/l HEPES buffer were used for all cultures. Non-adherent cells were removed by vigorous washing and the adherent cells were cultured for additional 48 hrs before use. L cell-conditioned medium (LCCM) was obtained from 4–5 day cultures of murine L929 cells, and was diluted 1:5 in DMEM before use.

IFN-induction. Macrophages were exposed to the indicated compounds for 1 hr, followed by washing twice with fresh media. The cells were incubated for 7 hrs unless indicated otherwise. The culture supernatants were harvested, separated into three pools, and frozen until assayed. When polymyxin B was used, it was premixed with the indicated IFN-inducer and incubated at room temperature for 1 hr before use.

IFN assay. Culture supernatants were assayed for IFN activity using a viral plaque-reduction assay. Briefly, monolayers of L929 cells in 96 well dishes were exposed to serial dilutions of culture supernatants for 6 hrs. The media were removed, and fresh media containing 50–75 PFU of vesicular stomatitis virus were added. After 1 hr, the virus was replaced with an overlay consisting of 0.5 % methyl cellulose in DMEM, supplemented with 2 % FBS, and the cells were incubated until plaques formed. The monolayers were stained with crystal violet, and the plaques were counted. The IFN titer was calculated to be the reciprocal dilution showing a 50 % reduction in plaque number compared to controls.

Macrophage activation by peroxidases and glycosylated BSA. Macrophage-mediated cytotoxicity was measured as described previously (Wei *et al.*, 1986). Breifly, macrophages were cultured in 96 well dishes at a concentration of 10^5 cells/well. The cells were exposed to the indicated compounds for 1 hr, and washed twice before the addition of $5x10^3$ 3T12 cells. The cells were incubated for 48 hrs, fixed with formalin, and stained with methylene blue. The stain was eluted with 0.1 N HCl, and optical densities (OD) were measured using a Dynatech microtiter plate reader. Percent cytotoxicity was calculated as follows: the OD of wells containing macrophages alone were subtracted from the OD of wells containing macrophages and target cells. The differences between six or more wells were used to calculate the mean OD (MOD) value for each treatment. The value obtained from untreated controls were considered as 100 %. Percent cytotoxicity was calculated using the following formula:

$$1 - \frac{\text{MOD of treated cultures}}{\text{MOD of control cultures}} \times 100$$

Results

Induction of IFN by HRP

Exposure of peritoneal macrophages to HRP resulted in the production of IFN in a dose-dependent manner as shown in Fig. 1. IFN activity was detectable in culture supernatants within 4 hrs after exposure to HRP, with peak activity occurring by 8 hrs (Fig. 2). Pretreatment of the macrophages for 1 hr with actinomycin D (1 μg/ml) completely abolished the IFN response. In addition, anti-

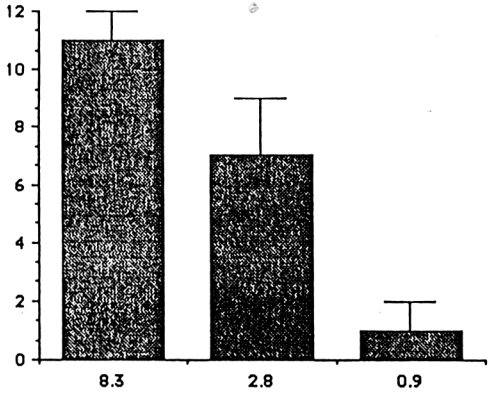

Fig. 1
IFN production by C57BL/6 macrophages exposed to HRP
Abscissa: HRP concentration in μmol/l. Ordinate: IFN titer in U/50 μl. Macrophages were exposed to HRP for 1 hr, washed twice, and incubated for 7 hrs. IFN activity was measured using a viral plaque-reduction assay, with the titer being the reciprocal dilution causing a 50 % reduction in plaque number. Histograms represent the mean IFN titer of triplicates assayed ± SD.

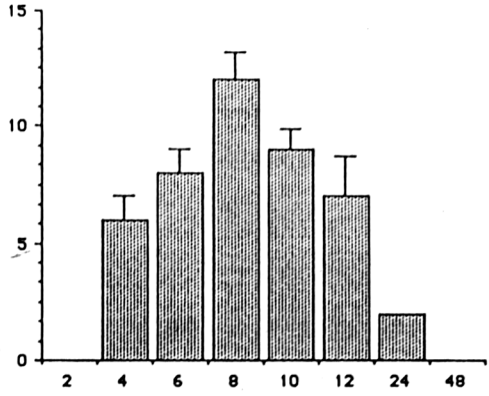

Fig. 2
Kinetics of IFN production by C57BL/6 macrophages exposed to HRP
Abscissa: time after exposure to HRP in hrs. Ordinate: IFN titer in U/50 μl. Macrophages were exposed to 4.15 μmol/l HRP for 1 hr and washed twice. Culture supernatants were harvested at the indicates times. IFN activity was measured using a viral plaque-reduction assay, with the titer being the reciprocal dilution causing a 50 % decrease in plaque number. Histograms represent the mean IFN titers of triplicates tested ± SD.

sera against murine IFN-beta completely blocked IFN activity in the culture supernatants (data not shown).

Culturing of macrophages in DMEM supplemented with 20 % LCCM enhanced the IFN response to HRP by as much as 12-fold (Table 1). The IFN response to LPS and poly I:C was also increased. Since LCCM increased the sensitivity of the macrophages to IFN induction, it was used in all the subsequent experiments.

Induction of IFN by glycosylated BSA

To determine whether other glycoproteins might induce IFN, a series of BSA glycoconjugates were selected based on their differing abilities to bind to the MFR, since this receptor has been demonstrated to participate in the

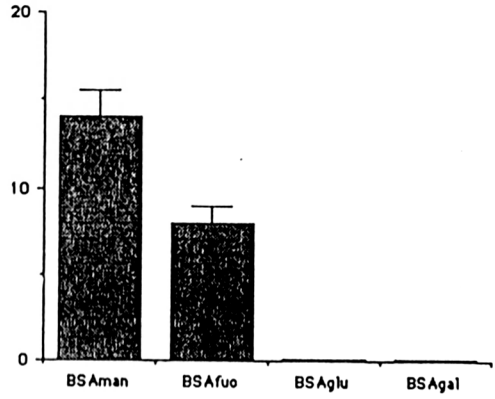

Fig. 3
IFN production by C57BL/6 macrophages exposed to BSA glycoconjugates
Abscissa: glycosylated BSA. Ordinate: IFN titer in U/50 μl. Macrophages were exposed to the indicated compound at a concentration of 100 μg/ml for 1 hr, washed twice, and incubated for 7 hrs. IFN activity was measured using a viral plaque-reduction assay, with the IFN titer being the reciprocal dilution causing a 50 % decrease in plaque number. Histograms represent the mean IFN titer of triplicates tested ± SD.

Table 1. Comparison of the IFN response of thioglycollate-induced C57BL/6 macrophages cultured in DMEM or LCCM

Inducer	Concentration	IFN titer	
		DMEM	LCCM
HRP	7.5 μmol/l	11 ± 1	50 ± 2
	2.5 μmol/l	7 ± 2	23 ± 5
	0.83 μmol/l	1 ± 1	12 ± 2
Poly I:C	10 μg/ml	113 ± 15	> 256
LPS	10 μg/ml	< 2	26 ± 1

Macrophages were exposed to the indicated inducer for 1 hr, washed twice, and incubated for 7 hrs. IFN titers are expressed as U/50 μl ± SD. Each value represents the mean titer of triplicates tested.

uptake of HRP (Stahl *et al.*, 1980). Mannosylated and fucosylated BSA, used at 100 μg/ml, induced IFN synthesis, while glucosylated or galactosylated BSA did not (Fig. 3). Since these data suggested that engagement of the MFR may be responsible for IFN-induction by these glycoproteins, attempts were made to block IFN induction by mannose (100 mmol/l) or yeast mannan (2 mg/ml). Neither substances were able to block IFN induction by HRP (data not shown).

The role of LPS in IFN-induction by glycoproteins

Polymyxin B at 10 ng/ml completely blocked IFN-induction by HRP or LPS (Table 2). The response of macrophages to 10 μg/ml poly I:C was unaffected by polymyxin B, demonstrating the specificity of polymyxin inhibition for LPS. In addition, C3H/HeJ macrophages failed to produce IFN in response to HRP or

Table 2. The role of LPS in interferon induction by peroxidases

	C57BL/6		C3H	
	Control	Polymyxin B	HeJ	HeN
HRP (4.15 μmol/l)	21 ± 2	< 2	< 2	6 ± 1.5
Poly I:C (10 μg/ml)	149 ± 37	163 ± 40	191 ± 15	136 ± 18
LPS (10 μg/ml)	13 ± 4.5	< 2	< 2	6 ± 1.5

Macrophages were exposed to the indicated inducer for 1 hr, washed twice, and incubated for 7 hrs. Where indicated, the samples were pre-mixed with polymyxin at a final concentration of 10 ng/ml prior to use. IFN titers are expressed as U/50 μl ± SD. Each value represents the mean titer of triplicates tested.

LPS, while C3H/HeN macrophages did produce IFN, but at lower levels than seen with C57BL/6 macrophages. These results indicate that IFN induction by peroxidase is dependent on LPS. However, the amount of LPS present in the peroxidase and glycosylated BSA preparations was insufficient to account solely for the IFN responses observed, since HRP contained ≤ 0.38 ng of LPS/100 μg protein.

Macrophage activation by peroxidases and glycosylated BSAs

The ability of HRP to activate C57BL/6 macrophages to the cytotoxic state has been reported previously (Wei *et al.*, 1986). For this reason, the ability of other glycoproteins to function in a similar manner was investigated, with the results illustrated in Table 3. Macrophage activation studies were performed in DMEM, since LCCM was found to cause significant spontaneous cytotoxicity. C3H/HeJ macrophages exposed to either mannosylated BSA or HRP for 1 hr exhibited significant cytotoxicity against 3T12 target cells (12 % and 32 %, respectively) ($p \leq 0.001$). Exposure of macrophages to other BSA glycoconjugates or LPS failed to induce significant cytotoxicity.

Discussion

The studies described herein utilized thioglycollate-induced peritoneal macrophages. These cells are considered to be inflammatory macrophages, which do not spontaneously secrete IFN, nor express spontaneous cytotoxicity against transformed cells (Adams and Marino, 1984). The macrophages used in studies on IFN induction were cultured in LCCM which is a source of colony stimulating factor-1 (Stanley and Heard, 1977). This medium was used since it was found to enhance the IFN response to a variety of IFN inducers. This enhancement did not appear to be due to division of macrophages *in vitro*, since

Table 3. Activation of C3H/HeJ macrophages by mannosylated and fucosylated glycoproteins

Inducer	Concentration	MOD	Cytotoxicity (%)
HRP	4.15 μmol/l	0.53 ± 0.055	32
BSAman	100 μg/ml	0.59 ± 0.052	12
BSAfuc	100 μg/ml	0.64 ± 0.044	3
BSAgal	100 μg/ml	0.68 ± 0.077	0
BSAglu	100 μg/ml	0.63 ± 0.056	0

Macrophages were exposed to the indicated compounds for 1 hr, and washed twice before addition of 3T12 target cells at an E:T ratio of 20:1. The cells were incubated for 48 hrs, fixed, stained with methylene blue, and the OD at 560 nm was measured. Cytotoxicity is expressed as a percent of untreated controls. Each value represents the MOD from at least six replicates.

no mitotic figures were observed in Giemsa-stained macrophages cultures after 48 hrs in culture roteins induced IFN from murine C57BL/6 and C3H/HeN macro.

The present study demonstrates that mannosylated or fucosylated glycoproteins induced IFN from murine C57BL/6 and C3H/HeN macrophages *in vitro*. IFN-induction by these glycoproteins was dose-dependent (data for BSA glycoconjugates not shown). Peritoneal macrophages express a MFR which binds mannose or fucose-terminal glycoproteins with high affinity, resulting in endocytosis of these ligands (Stahl *et al.*, 1980; Shepherd *et al.*, 1982). Indeed, HRP has been used extensively in the study of endocytosis via the MFR. It is proposed that engagement of this or a similar scavenger receptor may also be responsible for the production of IFN and macrophages activation. However, attempts to block this activity with mannose or mannan were unsuccessful. The MFR does not bind monosaccharides with high affinity compared to polysaccharides and glycoproteins, and might not be expected to block HRP or glycoBSA binding. Yeast mannan would be expected to successfully block both IFN and cytotoxicity induction. It is possible that the presence of excess mannan does not completely block the binding of the glycoconjugates, allowing sufficient binding of the glycoproteins to trigger the observed responses. Alternatively, mannan may bind to sites on the MFR different from those bound by glycoproteins. It has been shown that low concentrations of mannose enhance the binding affinity of the MFR, suggesting that more than one binding site exists on the MFR, and that a cooperative effect on subsequent binding occurs (Kaplan and Buys, 1985). It is intriguing that LPS was required for IFN induction via this pathway. It is possible that binding of these glycoproteins results in increased responsiveness to LPS-mediated IFN-induction. In other words, engagement of the MFR may lower the threshold for LPS-mediated IFN induction. Most enveloped viruses possess spike glycoproteins which bind to cellular receptors to facilitate virus entry and infection. In the case of human immunodeficiency virus, the spike glycoproteins are mannosylated (Geyer *et al.*, 1988). It is suggested that IFN synthesis may be a consequence of this mode of virus entry into macrophages.

Macrophages exposed to HRP or BSAman also acquired the ability to lyse 3T12 cells. In contrast to IFN-induction, however , macrophage activation was LPS-independent, since LPS-resistant C3H/HeJ macrophages were used in these studies. It appears that IFN induction and macrophage activation by these substances occurs via separate pathways. Previous studies demonstrated that macrophage activation *in vitro* occurred in a stepwise fasion which required LPS (Adams and Marion, 1984; Pace *et al.*, 1985). The present study demonstrates that mannosylated glycoproteins can activate macrophages independently of LPS. These results are significant since peroxidases are found in peripheral blood granulocytes (myeloperoxidase) and eosinophils (eosinophil peroxidase). Myeloperoxidase has been shown to be endocytosed by macrophages during inflammatory responses (Shellito *et al.*, 1987; Heifets *et al.*, 1980), and it is possible that this could result in the regulation of macrophage func-

tions *in vivo*. Preliminary evidence suggests that exposure of C3H/HeJ macrophages to human myeloperoxidase results in the expression of cytotoxicity; work in this laboratory is currently investigating this activity.

Malignant tumours often contain large numbers of leukocytes (Eccles and Alexander, 1974; Evans, 1972). In addition, neopterin, which is a marker for activated macrophages, is often present in the serum of individuals with malignant tumours (Huber *et al.*, 1984). It is felt that endogenously-derived peroxidases might serve to activate macrophages in an affort to clear neoplastic cells *in vivo*.

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