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Effect of interferon-gamma priming on the activation of murine peritoneal macrophages to tumouricidal state by cisplatin, IL-1, and tumour necrosis factor (TNF): production of IL-1 and TNF

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SUMMARY

The effect of interferon-gamma (IFN-γ) priming of murine peritoneal macrophages on the activation to tumouricidal state by cisplatin, lipopolysaccharide (LPS)-IL-1 and TNF was investigated. Cisplatin-, LPS-, IL-1- or TNF-treated IFN-γ-primed macrophages showed significantly enhanced tumouricidal activity and binding to tumour cells, compared with unprimed treated or untreated macrophages. Macrophages treated with cisplatin, LPS, IL-1 and TNF produced released and membrane-associated IL-1 and TNF activity which was significantly enhanced after priming with IFN-γ. These observations suggest the use of IFN-γ along with these biological response modifiers in designing immunotherapeutic protocols for treatment of malignancy.

Keywords interferon-gamma macrophages cytotoxicity IL-1 tumour necrosis factor

INTRODUCTION

Although macrophages are considered to be important effector cells in the host defence against neoplasia [1], little is known about the underlying cellular events involved in macrophage activation for tumour cytotoxicity. There is now definitive evidence that interferon-gamma (IFN-γ) is one of the important naturally occurring cytokines that contributes to or participates in the acquisition of the tumouricidal state by macrophages [2]. IFN-γ has been widely recognized as a potent macrophage-activating factor (MAF). Upon exposure to IFN-γ, macrophages develop the capacity to secrete reactive oxygen intermediates [3,4], extracellular enzymes [5], monokines [5,6] and cytotoxic molecules [5,7,8], as well as non-specifically to kill obligate or facultative intracellular micro-organisms [9] and a variety of neoplastic cells [2,10–12].

However, the mechanisms and kinetics of macrophage activation for a variety of biological activities have not yet been fully established [13]. Current understanding of the induction of murine macrophage tumour cytotoxicity suggests there are two alternative pathways. The first pathway requires two signals, distinguished operationally as 'priming' and 'trigger' signals; a priming signal is necessary to render macrophage responsive to a trigger signal [14,15]. So far IFN-γ is the only characterized lymphokine that provides the priming signal to macrophages for lysis of tumour targets [10,15,16]. A small amount of endotoxin is commonly used for *in vitro* studies to provide the trigger signal, but other lymphokines, such as TNF and granulocyte/macrophage colony-stimulating factor (GM-CSF) have also been demonstrated to trigger primed macrophages for cytotoxicity [17–20]. The second pathway requires only a single signal. Several lymphokines have recently been demonstrated to activate macrophages for tumour cytotoxicity via this pathway, including GM-CSF, IL-4, IL-1, TNF and IFN-γ itself [2,8,21–23].

Cisplatin is a potent anti-tumour compound which has been used successfully against several tumours in humans and animals [24]. We have reported that murine macrophages treated with cisplatin show enhanced capacity to lyse tumour cells *in vitro* [25,26] and also result in increased production of IL-1 and TNF [27], reactive oxygen metabolites [28], lysozyme and arginase [29]. Cisplatin can also activate human monocytes [30,31] and up-regulate IL-2-induced lymphokine-activated killer (LAK) activity in human peripheral blood mononuclear cells [32]. These studies clearly demonstrated that cisplatin may be used not only as a chemotherapeutic agent in cancer treatment but also as a potent monocyte–macrophage-activating agent.

The mechanism of the tumouricidal activity of the macrophages activated after priming with IFN-γ is not clearly understood. Moreover, the production of cytotoxic monokines by IFN-γ-primed murine macrophages in response to different trigger signals, particularly with cisplatin, is to date not clearly established. Therefore, the present investigations were undertaken to study the production of soluble and membrane associated forms of IL-1 and TNF by *in vitro* IFN-γ-primed macrophages in response to cisplatin, LPS, IL-1 and TNF. We also report the effect of IFN-γ priming on the tumouricidal activity and tumour cell binding to activated macrophages.

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MATERIALS AND METHODS

Mice

Inbred strain of healthy C3H/He male and female mice aged 8–10 weeks were used for obtaining peritoneal macrophages.

Tumour cells

L929 and YAC 1 cells were obtained from the National Tissue Culture Facility, Pune, India. They were maintained in tissue culture medium RPMI 1640 supplemented with 10% fetal calf serum (FCS). For cytotoxicity and TNF bioassay the cells in their exponential growth phase were used. Dalton's lymphoma (DL) cells (ascites) are maintained in our laboratory by a serial transplantation in C3H/He mice.

Reagents and culture media

Tissue culture medium RPMI 1640 and IFN-γ were purchased from Sigma Chemical Co. (St Louis, MO). The media used for all cell culture were supplemented with 20 μg/ml of gentamycin, 100 U penicillin and 10% heat inactivated FCS (Sera Lab, UK). ^{3}H-thymidine (^{3}H-TdR) was obtained from Bhabha Atomic Research Centre (Bombay, India). Recombinant human preparations of IFN-γ, IL-1 and TNF were obtained from Collaborative Research (Bedford, MA). All the reagents were free from endotoxin contamination, assayed by *Limulus amebocyte* lysate assay kit (sensitivity limit $<0{\cdot}05$ ng/ml).

Isolation and activation of macrophages

Macrophage monolayers were prepared as described previously [25] by peritoneal lavage of C3H/He mice. For priming macrophage monolayers ($1{\cdot}5 \times 10^6$ cells/well) were incubated with medium alone or medium containing IFN-γ. Incubation of macrophage monolayers for 4 h with IFN-γ at a dose of 10 U/ml was found to be optimal for priming. The macrophage monolayers were then washed with warm serum-free medium and then incubated with medium alone or containing LPS, IL-1, TNF, IL-2, or cisplatin for 24 h at various doses. After treatment these macrophage monolayers were washed and used for assaying macrophage-mediated cytotoxicity and for expression of IL-1 and TNF activities.

Macrophage-mediated cytotoxicity

Cytotoxicity was assayed by measuring release of radioactivity as described [25]. Target cells (DL and YAC 1) in exponential growth phase were incubated in medium containing 1 μCi/ml of ^{3}H-TdR. After 24 h of incubation, cells were washed and used as target cells in the cytotoxicity assay. Labelled target cells were plated into wells containing treated or untreated macrophage monolayers at an effector-to-target (E:T) cell ratio of 20:1. After 24 h of co-incubation, cell-free culture supernatants were harvested and an aliquot was counted for radioactivity in liquid scintillation counter (LKB, Bromma, Sweden). The percentage of macrophage-mediated cytotoxicity was calculated using the formula:

$$100 \times \frac{E-S}{T-S}$$

where E represents the radioactivity released in culture wells containing effector plus target cells; S represents spontaneous release, i.e. radioactivity released in cultures of target cells alone; and T represents total release, i.e. radioactivity released from target cells lysed with 1 *N* NaOH.

Spontaneous release was always $<10\%$ of total release.

Macrophage target cell binding assay

The binding assay was carried out as described [25]. Treated or untreated macrophage monolayers were washed and incubated in fresh medium containing ^{3}H-TdR-labelled target cells in an E:T ratio of 20:1. After 2 h of incubation the macrophage monolayers were washed two or three times with serum-free medium and digested with 0·25% SDS. The lysates were assayed for residual radioactivity in liquid scintillation counter. The target cell binding was calculated by the formula:

$$\frac{E}{T} \times 100$$

where E represents ct/min bound to monolayers; and T represents total ct/min of added target cells.

Assay of TNF activity

Cell-free culture supernatants obtained from treated or untreated macrophages were assayed for released TNF activity. Untreated or treated macrophages were fixed with 1% paraformaldehyde (PFA) for the assay of membrane-associated TNF activity. TNF activity was assayed by a modification of method of Flick & Gifford [33] using ^{3}H-TdR-labelled L929 cells; 3×10^4 labelled L929 cells in complete medium containing actinomycin D (2 μg/ml) were added to each well of 96-well flat-bottomed culture plate along with test supernatants of PFA-fixed macrophage monolayers. After 18 h of incubation at 37°C the monolayers were washed three times with serum-free medium and viable adherent cells were lysed with 1% SDS (w/v). Release of radioactivity from the lysed cells was counted in liquid scintillation counter. The TNF activity was expressed in terms of percentage cytotoxicity, which was calculated using the formula:

$$100 \times \frac{C-T}{C}$$

where C represents ct/min in target cells incubated with medium alone; and T represents ct/min in target cells incubated with test sample.

Assay of IL-1 activity

IL-1 activity was assayed by standard thymocyte proliferation assay as described by Mizel [34]. Thymocytes obtained from 4–8-week-old C3H/HeJ mice were incubated at a concentration of $1{\cdot}5 \times 10^6$ cells/well in 96-well culture plate with medium containing suboptimal dose of concanavalin A (Con A) (1 μg/ml) and 2-mercaptoethanol (2×10^{-5} M) along with PFA-fixed macrophages or macrophage culture supernatants. The cultures were incubated for 72 h. Eighteen hours before the termination of incubation period, cultures were pulse labelled with 0·5 μCi/well of ^{3}H-TdR. At the end of the incubation period the cells were washed three times with serum-free medium and lysate was prepared with 1% SDS (w/v). One-hundred microlitres of aliquot of the lysate were counted for radioactivity. IL-1 activity was expressed as the ^{3}H-TdR incorporation in ct/min.

Table 1. Effect of IFN-γ priming on the activation of macrophages to tumouricidal state by cisplatin (CP), LPS, IL-1 and TNF

	Cytotoxicity (%)			
	DL cells		YAC 1 cells	
Treatment	Unprimed	Primed	Unprimed	Primed
Medium	4·5±0·1	6·8±0·5	3·9±0·2	7·5±0·2
CP 1 μg/ml	12·2±0·8	49·7±0·5*	10·9±0·5	40·8±3·9*
2 μg/ml	14·7±0·8	51·4±3·1*	15·3±2·8	54·0±2·6*
10 μg/ml	46·8±3·9	47·9±4·0	51·2±2·9	54·3±1·8
LPS (1 μg/ml)	9·7±0·1	38·9±2·9*	12·2±1·0	41·5±2·7*
IL-1 (10 U/ml)	20·0±1·3	37·8±2·8*	17·2±1·6	40·7±3·9*
TNF (100 U/ml)	15·4±0·4	32·9±1·4*	18·3±1·7	37·0±3·2*

Murine macrophages were incubated in medium alone or medium containing 10 U/ml of IFN-γ for 4 h. After priming, the monolayers were washed and treated with or without CP, LPS, IL-1 or TNF for 24 h. Macrophage monolayers were washed and assayed for cytotoxicity against DL and YAC 1 cells. Values shown are means of cytotoxicity ± s.d. and are representative of three independent experiments with similar results. * $P < 0.05$; significantly different from the respective controls.

Table 2. Effect of IFN-γ priming on the binding pattern of macrophages activated with different biological response modifiers to tumour cells

	Binding of DL cells (%)	
Treatment	Unprimed	Primed
Medium	3·8±0·0	4·8±0·3
Cisplatin (1 μg/ml)	11·2±0·8	37·0±2·8*
LPS (1 μg/ml)	12·8±0·9	41·4±2·8*
IL-1 (10 U/ml)	15·3±1·2	42·1±1·9*
TNF (100 U/ml)	21·0±1·8	41·3±4·0*

Primed or unprimed macrophages were incubated with medium alone or medium containing cisplatin, LPS, IL-1 or TNF and binding assay was performed. Values are means of % binding ± s.d. and are representative of three independent experiments with similar results. * $P < 0.05$; significantly different from the respective controls.

Statistical analysis

The statistical significance of difference between test groups was analysed by Student's *t*-test (two-tailed). All experiments were done each in triplicate and repeated at least three times.

RESULTS

Effect of IFN-γ priming on the tumouricidal activity of macrophages

Murine peritoneal macrophages were incubated in medium alone or medium containing IFN-γ, 10 U/ml for 4 h, which was observed to be optimal for priming (data not shown), and subsequently treated with or without different biological response modifiers (BRM) for 24 h as indicated in Table 1. The cytotoxicity was assayed against fresh allogeneic DL target cells and YAC 1 tumour cell line. Untreated macrophages were not tumouricidal. Similarly, IFN-γ-primed untreated macrophages showed little cytotoxicity against both tumour target cells. Unprimed macrophages on treatment with LPS (1 μg/ml), IL-1 (10 U/ml), and TNF (100 U/ml) for 24 h showed enhanced tumouricidal activity against both DL and YAC 1 tumour cells. Similarly, the macrophage cytotoxicity was enhanced when the unprimed macrophages were treated with cisplatin at a dose of 10 μg/ml, whereas lower doses of cisplatin induced little cytotoxicity. This cytotoxicity was significantly augmented when the macrophages were first primed with IFN-γ and then treated with these BRM. The enhancing effect of IFN-γ priming was apparent only at lower doses of cisplatin (Table 1). Therefore, in all subsequent experiments for studying the effect of IFN-γ priming we used cisplatin at a lower dose of 1 μg/ml for activation of macrophages. The increased cytotoxicity of the treated macrophages might be attributed to enhanced binding of tumour cells to the activated macrophages. Therefore, next we examined the effect of IFN-γ priming on the pattern of binding of DL cells to treated or untreated macrophages. The results are shown in Table 2. Similar to the pattern of cytotoxicity, the binding of tumour cells to the macrophages on treatment with cisplatin (1 μg/ml), LPS (1 μg/ml), IL-1 (10 U/ml) or TNF (100 U/ml) was significantly enhanced after priming with IFN-γ. Untreated macrophages or IFN-γ-primed untreated macrophages showed comparatively less binding to tumour cells (Table 2).

Effect of IFN-γ priming on the expression of IL-1 by murine peritoneal macrophages

Culture supernatants of untreated macrophages did not enhance the proliferation of thymocytes in response to Con A. Similarly, culture supernatants obtained from macrophages primed with IFN-γ for 4 h and then incubated in medium alone did not affect the proliferation of thymocytes. Whereas, macrophages treated with LPS, IL-1, TNF, or cisplatin showed increased release of IL-1 activity into the culture supernatants, as revealed by enhanced thymocyte proliferation in response to Con A, which was significantly augmented in macrophages that were first primed with IFN-γ and then treated with these BRM. The membrane-associated IL-1 activity was assayed in PFA-fixed macrophages. Results are shown in Table 3. Primed or unprimed untreated macrophages showed little membrane-associated IL-1 activity.

Treatment of unprimed macrophages with LPS, IL-1, TNF, or cisplatin resulted in enhanced expression of membrane-associated IL-1 activity, which was significantly augmented by priming with IFN-γ (Table 3).

Table 3. Expression of IL-1 activity in IFN-γ-primed macrophages treated with different biological response modifiers

	Thymocyte blastogenesis (ct/min ± s.d.)			
	Culture supernatant		PFA-fixed cells	
Treatment	Unprimed	Primed	Unprimed	Primed
Medium	4500 ± 120	6018 ± 1007	3502 ± 560	5380 ± 890
Cisplatin (1 μg/ml)	13000 ± 1289	18810 ± 1200*	9900 ± 872	13380 ± 1134*
LPS (1 μg/ml)	23000 ± 1870	33810 ± 2987*	10180 ± 1100	18775 ± 1234*
IL-1 (10 U/ml)	18770 ± 1252	28770 ± 1782*	15892 ± 1357	20076 ± 1876*
TNF (100 U/ml)	17100 ± 1350	24707 ± 2172*	12860 ± 1109	18860 ± 1230*

Murine peritoneal macrophages were incubated in medium alone or primed with IFN-γ (10 U/ml) for 4 h and then after washing the monolayers were treated with or without LPS, IL-1, TNF, or cisplatin. After 24 h the monolayers were washed and incubated with fresh medium for 24 h; culture supernatant were then harvested and assayed for released IL-1 activity. The macrophage monolayers were fixed with PFA. Values are representative of three independent experiments with similar results. * $P < 0.05$; significantly different from the respective controls.

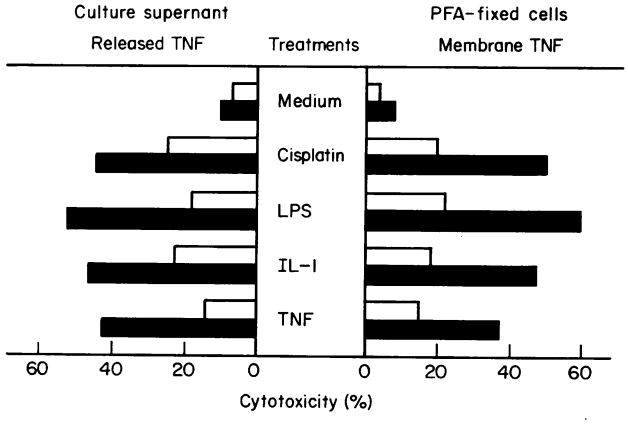

Fig. 1. Effect of IFN-γ priming on the expression of soluble and membrane-associated TNF by murine peritoneal macrophages. Murine peritoneal macrophages were incubated with medium alone (open bars) or containing IFN-γ (10 U/ml) for 4 h (solid bars). After washing the macrophage monolayers were treated for 24 h with medium alone or containing cisplatin (1 μg/ml), LPS (1 μg/ml), IL-1 (10 U/ml) or TNF (100 U/ml). Values are mean of triplicate cultures; s.d. in all experiments were consistently < 10% of the mean.

Production of TNF by IFN-γ-primed macrophages in response to different BRM

Culture supernatants obtained from unprimed or IFN-γ-primed and treated or untreated macrophages were assayed for released TNF activity. Unprimed or primed untreated macrophages showed negligible release of TNF activity (Fig. 1). Culture supernatants obtained from unprimed and LPS-, IL-1-, TNF-, or cisplatin-treated macrophages showed increased cytotoxicity against actinomycin D-treated L929 cells, which was significantly enhanced when the macrophages were primed with IFN-γ and then treated with these BRM (Fig. 1). PFA-fixed unprimed macrophages did not show TNF activity. PFA-fixed unprimed treated macrophages showed increased lysis of actinomycin D-treated L929 cells, which was significantly enhanced when macrophages were primed with IFN-γ and subsequently treated with LPS, IL-1, TNF, or cisplatin (Fig. 1).

DISCUSSION

Our findings show that priming of macrophages with IFN-γ significantly enhanced the macrophage-mediated cytotoxicity against DL and YAC 1 tumour target cells (Table 1), indicating that IFN-γ renders the macrophages more receptive to activation signals of cisplatin, LPS, TNF or IL-1. Our previous failure to detect priming by IFN-γ was probably due to the use of an optimal [35] rather than suboptimal dose of cisplatin. The major role for IFN-γ priming is to prepare the macrophages to respond to small amounts of second signals [10]. Similarly, in our study treatment of IFN-γ-primed macrophages with low doses of cisplatin induced significant macrophage-mediated tumouricidal activity. Such an increase in sensitivity has been one of the cardinal features used to characterize priming produced by IFN-γ. Thus our results indicate that IFN-γ-primed macrophages respond to suboptimal doses of activation agents. Cisplatin at higher doses is toxic to various cell types *in vitro* and *in vivo*. Since IFN-γ priming reduces the dose requirement of cisplatin for effective activation of macrophages, these observations are of particular significance and may have a far-reaching impact in designing therapeutic protocols with this chemotherapeutic drug.

It is well known that both IL-1 and TNF not only activate macrophage tumouricidal cytotoxicity, but also trigger the

release of more IL-1 and TNF [23]. Cisplatin-treated macrophages were also reported to express enhanced IL-1 and TNF activities [27]. Moreover, since IL-1 and TNF are also immunomodulators of the host defence against neoplasia and infection [23], we were interested to study the regulatory effect of IFN-γ priming on the production of IL-1 and TNF by macrophages activated with cisplatin, LPS, IL-1 or TNF. IFN-γ-primed macrophages did not produce IL-1 and TNF activity. Activation of IFN-γ-primed macrophages with cisplatin, LPS, IL-1 or TNF enhanced production of IL-1 and TNF compared with unprimed macrophages. Other investigators have shown that IFN-γ priming enhances the release of IL-1α and IL-1β and TNF activities by LPS-treated macrophages [36–38]. The mechanism of the regulation of IL-1 and TNF production by IFN-γ-primed macrophages is not clearly understood, but several possibilities can be considered. IFN-γ may act through a general mechanism in which sensitivity to signals inducing the synthesis and/or release of proteins is increased, or by a more specific mechanism in which the production of IL-1 and TNF are affected differentially as found in other systems [36–38]. The increased expression of IL-1 and TNF by cisplatin-, IL-1- or TNF-treated IFN-γ-primed macrophages may or may not follow the same mechanisms as described for LPS. Newton [39], reported that IFN-γ does not enhance monocyte sensitivity to LPS but amplifies the response to LPS for secretion of IL-1. Further, it is possible that IFN-γ acts to increase the number of cells responding to cisplatin, LPS, IL-1 or TNF, resulting in increased IL-1 and TNF secretion.

Global attempts are being made to develop effective immunotherapeutic protocols for treatment of malignancies. These observations suggest the possibility of using IFN-γ along with cisplatin, TNF or IL-1 in preclinical and clinical therapeutic trials.

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