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TUMOR NECROSIS FACTOR STIMULATES INTERLEUKIN-1 AND PROSTAGLANDIN E_2 PRODUCTION IN RESTING MACROPHAGES

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We have investigated the effect of tumor necrosis factor on the release of interleukin-1 and PGE_2 from murine resident peritoneal macrophages. Tumor necrosis factor causes an increase in the production of interleukin-1 and PGE_2 with a maximum induction for both noted at 5.9×10^{-8}M. While indomethacin decreased tumor necrosis factor induced PGE_2 production, this cyclooxygenase inhibitor augmented tumor necrosis factor induced interleukin-1 production. Our data suggests that tumor necrosis factor may be an important immunopotentiating agent in addition to its previously described cytolytic and metabolic activities.

A number of common biochemical and physiological alterations can be observed in animals responding to an in vivo challenge of bacteria or bacterial products. These derangements include an elevated body temperature (1), muscle catabolism (2), increase in acute phase proteins (3), immune effector cell activation (4,5), and hyperlipidemia (6). Current evidence has demonstrated that the above responses are attributable to monocyte/macrophage-derived peptides that are produced and released upon appropriate stimulation. One such peptide, tumor necrosis factor (TNF) , was originally described by Carswell as an oncolytic agent found in the serum of BCG immunized mice challenged with endotoxin (7). Although the initial interest focused on the cytotoxic effect of TNF, recent evidence has demonstrated that TNF and structurally homologous peptides, such as cachectin, must be considered as compounds that can stimulate

Abbreviations used in this paper: TNF, tumor necrosis factor; IL-1, interleukin-1; PGE_2, prostaglandin E_2; LPS, lipopolysaccharide.

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a variety of cellular responses associated with an immune event. These activities range from augmenting inflammatory cell activity by increasing neutrophil phagocytic and cytotoxic activity to suppressing the production of lipoprotein lipase resulting in the mobilization of energy reserves (8,9).

In the present paper we demonstrate the ability of TNF to stimulate the production of interleukin-1 (IL-1) and prostaglandin E_2 (PGE_2) from resident peritoneal macrophages. This additional, novel activity of TNF may contribute to macrophage-mediated immune reactions by serving as a signal responsible for the continued maintenance of the inflammatory reaction. These signals may provide a unique means for cells participating in local inflammatory lesions to communicate with other immune cells, resulting in amplified and/or modulated immune response.

MATERIALS AND METHODS

Animals - Female, specific pathogen-free C3H/HeJ, endotoxin resistant, and CBA/J endotoxin sensitive mice (Jackson Laboratories, Bar Harbor, ME) were used throughout the experiments. Mice were maintained under pathogen-free conditions and given food and water ad libitum.

Macrophage cultures - Mouse blood monocytes and resident peritoneal macrophages were obtained as previously described (10). Cells were washed, counted, and suspended to 1×10^6/ml RPMI with 5% fetal bovine serum (FBS) and 100 U penicillin/100 µg streptomycin/ml. One ml of the cell suspension was dispensed onto 35 mm sterile plastic culture dishes (Corning Glass Works, Corning, NY). After 2h incubation, (37°, 5% CO_2, 100% humidity) nonadherent cells were removed by two vigorous rinses.

The monolayers were overlaid with 1 ml of serum-free media (RPMI with antibiotics) plus various concentrations of recombinant TNF (specific activity 10^7 units/mg, Mw 17,000). Purified rTNF (lot #LYM12-072685C) and rabbit α human TNF neutralizing antibody (lot 57-030685) were provided as gifts by Dr. Leo S. Lin; Cetus Corp., Emeryville, CA. Endotoxin contamination of 1 µg/ml TNF was less than 150 pg/ml. Indomethacin (Sigma Chemical Co., St. Louis, MO) at a final concentration of 10^{-7} M and/or Salmonella lipopolysaccharide (LPS) (Sigma Chem. Co., St. Louis, MO) at 10 µg/ml were used in specified plates. Supernatants were collected after 16h incubation. The total number of adherent macrophages was quantitated by scraping the cells and counting in a hemocytometer. The adherent cells were >95% macrophages by morphology and phagocytic index.

Radioimminoassay for Prostaglandins E_2 - The macrophage culture medium was collected, centrifuged at 500 xg for 10 min and then subjected to radioimmunoassay for PGE_2 as described by Fitzpatrick et al. (11). Briefly, samples were extracted to remove protein and free fatty acids before addition of antibodies. Bound and free ligand were separated using dextran-coated charcoal. Sensitivity for PGE_2 was 8 picograms.

Interleukin-1 assay - Interleukin-1 was measured by the standard thymocyte proliferation assay (12). Briefly, 5×10^5 mouse thymocytes in 0.1 ml RPMI-10% FBS containing 2.5 µg/ml PHA (Burroughs Welcome, Research Triangle, NC) were distributed into each well of a 96-well sterile culture dish (Costar, Cambridge, Mass.). Equal volumes of serially diluted test supernate were then added to triplicate wells. Control wells contained no PHA. At 64h of incubation, the cells were pulsed with 0.5 µCi of tritiated thymidine then harvested

at 72h. Total thymidine incorporation was determined by liquid scintillation spectrophotometry. Augmentation of PHA-induced thymocyte proliferation indicated IL-1 activity. Data represents quantitation of PHA responses using 1:8 dilutions of macrophage culture supernatants.

Statistical analysis - The Student's t-test was used to compare control and experimental groups. Values of $p > .05$ were considered not significant.

RESULTS

Effect of TNF on IL-1 Production - Initially, we examined the effects of varying amounts of TNF to induce the release of IL-1 from resident peritoneal macrophage. Control culture supernatants revealed no IL-1 activity beyond media alone, while TNF caused statistically significant increase in IL-1 production (Fig. 1). A greater than 8-fold increase in the production of IL-1 by resident macrophages was observed when stimulated with 5.9×10^{-8}M TNF. Augmented IL-1 production by blood monocytes treated with the same doses of TNF was also observed, but the percent increase above control levels were significantly lower (data not shown). An augmentation of the TNF effect on resident peritoneal macrophages was observed with the cyclooxygenase inhibitor indomethacin. Treatment with 10^{-7}M indomethacin resulted in nearly a 2-fold increase

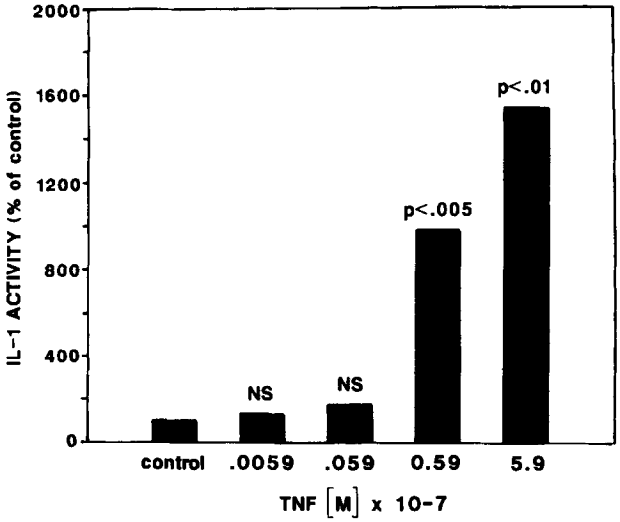

Figure 1. Effect of TNF on the production of IL-1 from resident peritoneal macrophages. Macrophage cultures (2.5×10^5/dish) were incubated for 16h with the TNF and supernatants were then tested for IL-1 activity. TNF alone had no effect on the proliferation assay.

Table 1. Effect of TNF in the presence of either LPS or 10^{-7}M indomethacin

Culture condition	^{3}HTdR incorporation (CPM)
Control	921 ± 101
LPS (10 μg/ml)	12,069 ± 1,050
TNF (5.9×10^{-8}M)	9,540 ± 1,125
TNF (5.9×10^{-8}M) + Indomethacin (10^{-7}M)	18,390 ± 2,350
TNF (5.9×10^{-8}M) + LPS (10 μg/ml)	23,510 ± 4,900

The above data is representative of quadruplicate experiments. Similar TNF-induced IL-1 production was observed with both CBA/J and C3H/HeJ macrophages. In the absence of macrophage supernatant, PHA alone gave 523 ± 250 cpm. In addition, 0.25 units of commercially available IL-1 (Cistron Technology, Pinebrook, N.J.) gave 9763 ± 1,610 cpm. To quantitate TNF activity, 5.9×10^{-8}M = 1.0 μg/ml = 10^{4} units/ml.

in TNF-induced IL-1 production (Table 1). The magnitude of the increase in TNF-induced IL-1 production was significant, as 5.9×10^{-8}M (1μg/ml) TNF induced similar levels of IL-1 production as 10 μg/ml of LPS. The addition of both LPS and TNF to the resident peritoneal macrophage cultures resulted in an additive effect on the production of IL-1 (Table 1); suggesting that resident peritoneal macrophages are susceptible to simultaneous stimuli that may enhance IL-1 production. Of particular interest was the additive effect of LPS and TNF on IL-1 production by C3H/HeJ, LPS resident mice. Although these mice showed a decrease in the release of IL-1 in response to LPS (10 μg/ml), the concomitant addition of LPS (10 μg/ml) plus TNF (1 μg/ml) resulted in an approximate 2-fold increase above TNF alone. In addition, TNF may serve as a potent stimulus for non-elicited resident macrophages and blood monocytes, since elicited macrophages are not nearly as receptive to TNF challenge. TNF alone had no effect on the thymocyte proliferation assay.

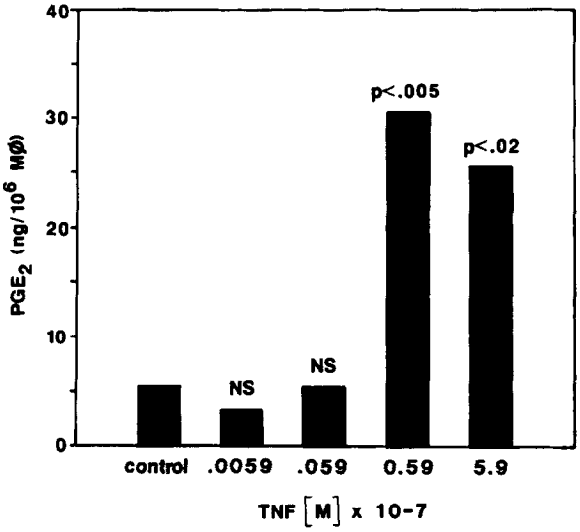

Figure 2. Levels of PGE_2 generated by resident peritoneal macrophages in response to graded doses of TNF. The concentration of PGE_2 represents the mean of 4 individual experiments.

Effect of TNF on PGE_2 Production. We next determined the effect of TNF on the production of PGE_2 by the cultured macrophages. As shown in Figure 2, TNF demonstrated a statistically significant increase in the production of PGE_2. The amount of TNF needed to induce PGE_2 production was nearly identical to that for TNF-induced IL-1 production, as PGE_2 production plateaued at a TNF concon-centration of 5.9×10^{-8}M. Indomethacin (10^{-7}M) treatment reduced the levels of TNF-induced PGE_2 production by 80-85%. (5.9×10^{-8}M TNF = 33 ng $PGE_2/10^6$ cells and 5.9×10^{-8}M TNF + 10^{-7}M indomethacin = 5 ng $PGE_2/ 10^6$ cells.) These data suggest that TNF-induced IL-1 production may be under the regulatory influence of specific cyclooxygenase derived metabolites.

Specificity of TNF Induced IL-1 Release. We next examined the specificity of the TNF response to document that the IL-1 release induced by TNF was specific and not solely related to endotoxin contamination (Table 2). Heating TNF at 56°C for 30 minutes destroyed the majority of TNF induced IL-1 activity, while endotoxin activity was unaffected. Heated TNF resulted in a greater than 80% reduction in TNF induced IL-1 release compared to unheated TNF, while heated and unheated LPS induced similar amounts of IL-1. Furthermore, addition

Table 2. TNF induced release of IL-1 is specific and not totally endotoxin dependent

Experiment	Culture condition	^{3}H TdR incorporation (cpm/10^6 cells)
1	TNF (1 μg/ml)	11,708 ± 1018
	LPS (10 μg/ml)	14,187 ± 938
	*heated TNF (5.9×10^{-8}M) (1 μg/ml)	2096 ± 344
	*heated LPS (10 μg/ml)	17,261 ± 10,597
2	TNF (2.9×10^{-7}M) (5 μg/ml)	13,181 ± 2201
	TNF (2.9×10^{-7}M) (5 μg/ml) + αTNF antibody†	1219 ± 284
3 (C3H/HeJ mice)	TNF (5.9×10^{-8}M) (1 μg/ml)	11,826 ± 1072
	LPS (1 μg/ml)	2742 ± 825

The above data is representative of triplicate experiments. Commercially available IL-1 (0.25 units; Cistron Technology, Pinebrook, N.J.) gave 6744 ± 1446. Control macrophage supernatants without an added stimulus gave 5-10% of the TNF stimulated supernatants. Neutralization of TNF activity by either heating at 56°C for 30 min* or adding neutralizing αTNF antibody† was verified by showing protection of L-929 cells from TNF induced cytolysis.

of specific αTNF neutralizing antibodies reduced TNF induced IL-1 production by 90% (Table 2). Lastly, peritoneal macrophages from LPS resistant mice (C3H/HeJ) responded to TNF stimulation with IL-1 release, while LPS was not as effective in inducing IL-1 from this strain of mouse.

DISCUSSION

It is well known that macrophages are capable of producing several important molecular signals during the initiation and maintenance of inflammatory reactions. Interleukins, prostaglandins, and tumor necrosis factor are all important members of a spectrum of highly refined mediators that may dictate the course of immune responses. Despite their potential importance in immune reactions, the bidirectional communication between these mediators has not been

described. In this paper, we present direct evidence that TNF can stimulate macrophages to produce IL-1, and that cyclooxygenase pathway inhibition can augment this effect. The latter observation is supported by the fact that TNF can stimulate PGE_2 production by cultured macrophages.

The mechanism by which TNF stimulates IL-1 production remains unclear. The effect is not simply due to the presence of LPS in the TNF preparations because: a) the magnitude of the IL-1 release induced by TNF is far greater than can be explained by low concentrations of LPS; b), the induction of IL-1 release by TNF was markedly decreased by heating or by the addition of αTNF neutralizing antibody; c), macrophages from endotoxin resistant mice released IL-1 in response to TNF; and d) TNF and LPS act in an additive manner to stimulate macrophages to produce more IL-1 than either substance alone. This synergistic activity may have important consequences in clinical states, such as sepsis, where both endotoxin and TNF may be present (13). Under such conditions these signals could act to accelerate the numerous metabolic and immunologic derangements that are associated with this malady. The augmented production of IL-1 by TNF and LPS could contribute to cachexia, by accelerating skeletal-muscle proteolysis (2); could contribute to physiologic changes, by inducing fever (1); and could contribute to immune alterations, by activating various inflammatory cells (4,5).

Our data may also serve as a model for the regulation of an additional macrophage-derived mediator by PGE_2. Recently, we have demonstrated that PGE_2 is an endogenous modulator of LPS-induced macrophage IL-1 production (10,14). Moreover, IL-1 possesses the characteristics of a hormone by inducing its own suppressing mechanism. The ability of TNF to induce PGE_2 production and augment IL-1 production in the presence of indomethacin suggests that this cyclooxygenase metabolite plays an important regulatory role with regard to an additional macrophage secretory product.

In conclusion, we provide evidence that TNF can induce IL-1 and PGE_2 production by resident peritoneal macrophages and the production of IL-1 is augmented by inhibition of the cyclooxygenase pathway of arachidonic acid

metabolism. These data support the notion that TNF is much more than an oncolytic agent and may play an important role in numerous macrophage-mediated events.

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