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Biologic and Immunohistochemical Analysis of Macrophage Interleukin-1α, -1β, and Tumor Necrosis Factor Production During the Peritoneal Exudative Response

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The present study examined changes in lipopolysaccharide (LPS)-induced interleukin 1 (IL-1) and tumor necrosis factor (TNF) production by murine peritoneal macrophages during the chronic exudative response to Freund's complete adjuvant (CFA). Macrophages were isolated by peritoneal lavage and adherence at intervals over a 32 day period following i.p. injection of CFA. Optimal culture conditions for IL-1 and TNF production were predetermined, and it was found that IL-1 production was profoundly impaired at densities of above 150 cells/mm², whereas TNF sythesis was more resistant to density effects. Using optimal conditions, we observed a sequential appearance of monokines. On day 0 there was minimal IL-1 production and no detectable TNF production. By days 4–7, IL-1 production reached maximum levels with a steady decline to baseline by day 32. TNF production steadily increased after day 2, reached maximal levels by days 16–20, and then partly declined by day 32. These findings were supported by kinetic analyses at specified days. When related to exudative events, it appeared that maximal IL-1 was associated with the recruitment stage of the reaction, whereas TNF production was associated with the established exudate. Immunohistochemical analysis revealed that TNF production could be related to the proportion of macrophages with cytoplasmic TNF expression. In contrast, IL-1α and -1β expression was comparable among populations with 85–100% of cells showing cytoplasmic expression 6 hr after LPS stimulus. Whereas cytoplasmic IL-1α persisted for the 18 hr study period, IL-1β disappeared from many adjuvant recruited cells. Our findings suggest that monokine production is orchestrated during macrophage recruitment and activation at sites of chronic inflammation.

Key words: monokines, immunohistochemistry, inflammation

INTRODUCTION

In recent years the monokines, interleukin-1 (IL-1) and tumor necrosis factor (TNF), have been the subject of intense investigation. Both of these polypeptide molecules are of relatively small molecular weight (15–18 kd) and are considered to have a variety of often overlapping effects on a number of target tissues [1–4]. Two structurally distinct forms of IL-1, designated α and β, have been identified and have similar biologic activities [5–7]. Many animal and human studies have shown associations of IL-1 and TNF with inflammatory responses [8–11], but there is little knowledge on the regulation and orchestration of their production during such responses.

In the present study, we approached this question by determining optimal culture conditions for monokine production and then establishing the sequential capacity of peritoneal macrophages to produce IL-1 and TNF during the exudative response to an injection of complete Freund's adjuvant (CFA). Our results indicate that augmented lipopolysaccharide (LPS)-induced IL-1 production occurs early, reaching maximal rates of synthesis from 4–7 days post-injection of CFA. In contrast, peritoneal macrophages did not acquire maximum capacity to produce TNF until 16–20 days after injection. When related to exudate events, IL-1 production was associated

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with the early recruitment phase of the exudate, whereas TNF production was maximal in the established exudate.

Immunohistochemical analysis of the cytoplasmic expression of TNF generally suggested that levels of biologically active material could be related to the proportion of macrophages synthesizing TNF. In contrast, analysis of IL-1α and -1β showed comparable cytoplasmic expression among all populations despite differences in secreted levels of bioactive material. Thus it appeared that the degree of IL-1 secretion was modified during the exudative response. There was some indication that IL-1β was cleared from adjuvant-elicited but not resident cells, supporting the notion that IL-1β is the dominant secreted IL-1 [7]. Taken together, our findings suggest that monokine production is indeed orchestrated during macrophage recruitment and activation, resulting in the sequential appearance and modified secretion of these factors.

MATERIALS AND METHODS

Animals

Female, CBA/J (Jackson Laboratories, Bar Harbor, ME) and CD1 Swiss albino (Charles River Laboratories Inc., Portage, MI) mice were maintained under specific pathogen-free conditions and provided with food and water ad libitum.

Macrophage Isolation and Culture

Peritoneal exudate cells were obtained by aseptic peritoneal lavage at designated intervals following intraperitoneal injection of 0.25 ml of complete Freund's adjuvant (CFA) (Sigma Chemicals, St. Louis, MO) that had been emulsified with an equal volume of normal sterile saline. The cells were washed by centrifugation and then suspended in RPMI (GIBCO, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Hazelton, Lenexa, KS), 2 mM glutamine, and 100 U penicillin/100 μg/ml. Cell yields were determined, and concentrations were adjusted to acheive optimal cell densities for monokine production, usually 0.5 to 1 × 10^6/ml. For density studies, plating concentrations were varied from 0.5 to 5 × 10^6 cells/ml. Macrophages were isolated by 2 hr adherence to 33 mm culture dishes (Corning Glass Works, Corning, NY) at 37°C in a 5% CO_2 humidified atmosphere. The nonadherent cells were removed by two vigorous washings with 1 ml of warm RPMI. The macrophage monolayers were then overlaid with RPMI-FBS containing 1 μg/ml LPS (*Escherichia coli* 011) (Sigma); controls contained no LPS. Supernatants were collected at designated intervals following LPS stimulation, centrifuged at 500*g* for 10 min to remove particulates, and then frozen at −20°C before monokine assay. Monolayers were scraped into 1 ml of PBS, and the number of cells was determined using a hemocytometer.

Exudate cell yields and peripheral blood leukocyte counts were determined for individual mice. Smears of peripheral blood and peritoneal exudates were also prepared and stained with a standard Wright stain for differential counting.

Interleukin-1 Assay

IL-1 was assayed by the standard thymocyte comitogen assay as modified from the procedure of Mizel et al. [12] and Koopman et al. [13]. Briefly, single cell suspensions of thymocytes were prepared from 5–7 week old CBA/J mice. The cells were washed and suspended in 5 × 10^6/ml of RPMI-RBS. The thymocytes (5 × 10^6) were cultured in 0.2 ml in 96-well flat-bottomed culture plates (Corning) in the presence of 1 μg/ml of purified phytohemagglutinin (PHA) (Wellcome Research Laboratories, Beckenham, England) and serial $\log_2$ dilutions of test supernate for 72 hr. The culture wells were each pulsed with 0.5 μCi of ^{3}H-methyl-thymidine (ICN, Irvine, CA) for the final 12 hr of culture. The cells were then harvested onto glass filters, and uptake was quantitated by scintillation spectrophotometry. IL-1 activity was quantitated by calculation of ½ maximal units [14]. Recombinant murine IL-1α and -1β standards were used as positive controls; approximately 50–100 pg were equivalent to ½ maximal unit.

Tumor Necrosis Factor Assay

The LM fibroblast cell line was used to measure levels of TNF in supernatants according to a modification of the procedure of Ruff and Gifford [15]. Fifty thousand LM cells in 0.1 ml RPMI-FBS were added to each well of a 96-well microtiter dish, and then serial $\log_2$ dilutions of test supernatant were added to each well. Actinomycin D was then added to each well to achieve a final concentration of 1 μg/ml. The dishes were then incubated for 18 hr at 37°C. After incubation the supernatants were discarded, and the remaining adherent viable cells were stained with 0.1 ml of cystal violet (0.2% in 2% ethanol) for 2 min. The microtiter dishes were rinsed 4 × with PBS, and the absorbance of each well was read at 600 nm with a microelisa Autoreader. TNF units were calculated from a standard curve generated with serially diluted recombinant TNF (Cetus Immune Laboratories, Palo Alto, CA).

Antisera Preparation

Anti-IL-1α, anti-IL-1β, and anti-TNFα were produced in goats or rabbits. Recombinant murine IL-1α, -1β (Pfizer Pharmaceuticals, Groton, CT), or TNFα were administered in multiple intradermal sites with Freund's complete adjuvant followed by an equivalent

boost 2 weeks later. The resulting anti-IL-1 antisera reacted with both 17–18 kd and 33 kd species in Western blot analysis of macrophage lysates. Specificity of the anti-IL-1 antisera was confirmed by both competitive inhibition and reciprocal absorption studies (in press). The specificity and properties of the anti-TNF antiserum have been described [16].

Immunohistochemistry

Macrophage monolayers were fixed for 5 min in 4% paraformaldehyde in PBS and rinsed twice with PBS. Prior to staining they were fixed for 3 min in absolute methanol. The slides were rinsed again with PBS and treated with a 3% hydrogen peroxide solution for 10 min to inactivate any remaining peroxidase activity. For IL-1α staining, the slides were blocked with a 1:50 dilution of normal rabbit serum for 10 min at 37°C, and then decanted and exposed to a 1:2,000 dilution of anti-IL-1α or a similar dilution of nonimmune goat serum. After 10 min incubation at 37°C, the slides were rinsed thrice with PBS, then overlaid with biotinylated rabbit anti-goat IgG (1:200) (Vector Laboratories, Burlingame, CA) and incubated another 10 min, followed by three additional rinses with PBS. The slides were next treated with peroxidase-labeled streptavidin (Sigma), incubated again, rinsed thrice, and then overlaid with substrate chromogen (3-amino-9-ethyl-carbazole) for 5 min at 37°C to allow for color development. Mayer's hematoxylin was used as a counterstain. Staining for IL-1β and TNF was similar except slides were blocked with goat serum and then treated with a 1:1,000 dilution of rabbit anti-IL-1β, rabbit anti-TNFα, or control nonimmune serum followed by biotinylated goat anti-rabbit IgG. The slides were then observed by light microscopy. Percent positive cells was determined by counting a minimum of 200 cells.

Statistics

Student's t test was used to determine differences between control and experimental groups. Values of $P > 0.05$ were considered not significant.

RESULTS

Effect of Cell Culture Density and LPS Concentration on Monokine Production

In order to compare macrophage populations with regard to their capacity to produce monokines, it was important to first establish optimal culture conditions. To approach this question, we examined two important variables, cell density per culture dish and concentration of the LPS stimulant. As shown in Figure 1, changing these conditions caused distinct effects on IL-1 and TNF production. Interleukin 1 production was very sensitive to cell density, with the optimum occurring only at lower densities (50–100 cells/mm^2). Also, IL-1 production was LPS-dose dependent only at lower cell densities. Tumor necrosis factor production was LPS-dose dependent at all cell densities. However, at the lower LPS concentration, increasing density resulted in reduced production per cell. In contrast to that for IL-1, this effect could be largely overcome by using a higher concentration of LPS (10 μg/ml). Thus, TNF production was more resistant to cell density effects in the presence of higher concentrations of LPS.

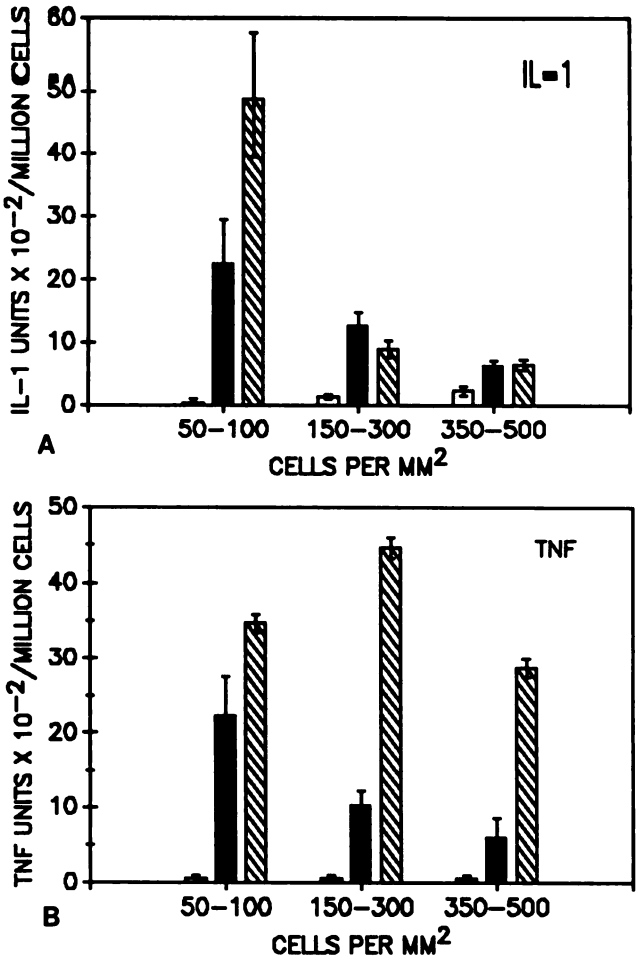

Fig. 1. Effect of cell culture density and LPS concentration on interleukin 1 (IL-1) (A) and tumor necrosis factor (TNF) (B) production. Peritoneal exudate cells were collected from CD1 mice 14 days after i.p. injection of complete Freund's adjuvant. Macrophage monolayers were prepared at varying densities and then cultured for 18 hr in the presence of no LPS (open bars), 100 ng/ml LPS (solid bars), or 10 μg/ml LPS. Supernatants were collected and assayed for IL-1 and TNF activity. Bars are mean ± SE of three determinations.

Time Course of Monokine Production During the Exudative Response

Figure 2 illustrates the dynamic changes in cell populations that occurred in the blood and peritoneum fol-

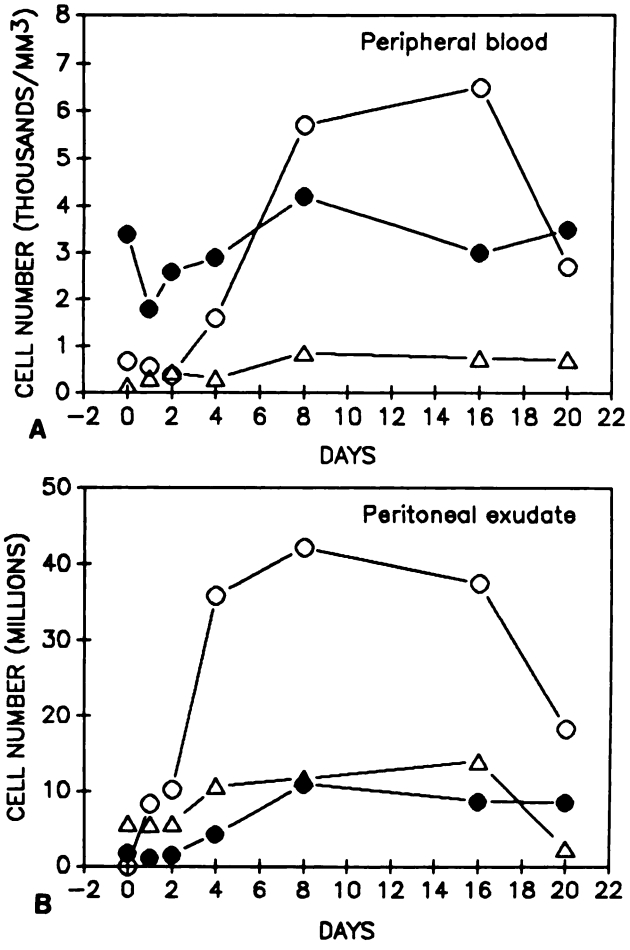

Fig. 2. Changes in peripheral blood (A) and peritoneal leukocyte populations (B) during the adjuvant-induced chronic peritonitis. Points represent mean of five mice.

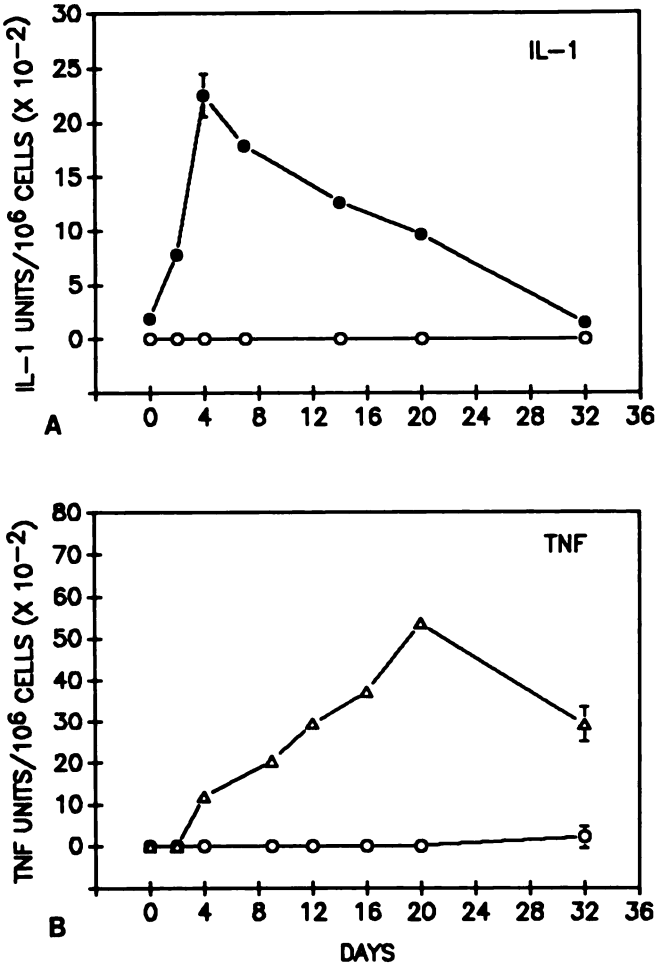

Fig. 3. Production of interleukin 1 (IL-1) and tumor necrosis factor (TNF) by cultured peritoneal macrophages during the chronic exudative response. Macrophage monolayers were prepared at optimal densities at the indicated intervals following exudate induction. Supernatants were collected after 18 hr of culture in the presence or absence of LPS and then assayed for IL-1 and TNF activity. A: IL-1 activity. Open circles, no LPS; closed circles, 1 μg/ml LPS. B: TNF activity. Open circles, no LPS; triangles, 1 μg/ml LPS. Points are mean ± SE of three to six determinations.

lowing an intraperitoneal injection of complete Freund's adjuvant. In the blood there was a transient decrease in lymphocytes in the first 24 hr, which steadily recovered over the subsequent 7 days. A significant polymorpholeukocytosis was evident by day 4, followed by a monocytosis on day 8. By day 20, PMNs began to decrease, but monocytes were still elevated. In the peritoneal exudate there was an intial influx of PMNs detected on day 1. By day 4 there was a dramatic increase in PMNs and a significant accumulation of mononuclear phagocytes and lymphocytes. Throughout the study period, PMNs were the dominant component of the exudate. By day 20, the phagocyte populations showed declines indicating the resolution phase of the exudate.

In view of the dynamic events occurring in inflammatory cell populations, it was of interest to determine if monokine production could be related to immunopathologic events. Indeed, when we examined macrophages isolated at intervals following adjuvant injection, there was clearly a changing capacity of cells to produce these monokines (Fig. 3). On day 0 IL-1 production by resident peritoneal macrophages was minimal but detectable. Following CFA injection, there was a rapid augmentation of IL-1-producing capacity, which reached maximal levels by days 4–7. This capacity steadily declined thereafter, reaching baseline levels by day 32. In contrast, TNF production was absent at day 0, and there was a slower rise in production capacity, with maximal levels being acheived by days 16–20. By day 32 there was a significant decline in TNF production, but this was still elevated compared to day 0 levels. Thus, IL-1 pro-

TABLE 1. Effect of Anti-TNF Immunoabsorption on TNF and IL-1 Supernatant Bioactivity

	Monokine activity (units/ml)[a]	
Column specificity	TNF	IL-1
Control[b]	78 ± 37	350 ± 135
Rabbit anti-murine TNF	0 ± 0	417 ± 101

[a]Mean ± SD of three determinations.
[b]Nonimmune rabbit IgG.

duction was associated with the elicitation/recruitment phase of the response, whereas TNF production was more characteristic of the established exudate. Intriguingly, the time of maximal IL-1 production corresponded to the time of lymphocytosis and lymphocyte accumulation.

Since IL-1 activity declined as TNF activity increased, it was important to determine whether or not TNF was interfering with the IL-1 bioassay. To test this possibility supernatants prepared from day 14 macrophage cultures were passed through control or anti-murine TNF immunoaffinity columns and then tested for both IL-1 and TNF activity. As shown in Table 1, IL-1 activity was not significantly changed after complete removal of TNF activity. Hence, TNF did not appear to interfere with IL-1 assessment.

Kinetics of Monokine Production During the Exudative Response

The above studies represented a single point (18 hr) analysis of TNF and IL-1 levels in macrophage culture supernatants and may not fully reflect the capacity of the cells to produce these monokines. Therefore, kinetic analyses were performed on cultures prepared at intervals following CFA injection. As shown in Figure 4, the observed rates of IL-1 production supported the initial findings. The rate and degree of IL-1 production increased after day 0, was greatest for macrophages from day 7 exudates, and then partially declined in day 14 cultures. It should be noted that for all cultures, the maximum rate of production occurred from 3 to 6 hr after stimulus. These kinetics correlated well with our previously published kinetic analysis of IL-1α and -1β mRNA accumulation, which reached a maximum at 6 hr and decayed thereafter [17].

Figure 5 shows a similar analysis of TNF production. No detectable TNF was produced early, at 0–2 days. Subsequently, by day 4 there was significant TNF production, but this increased to greater levels by day 14. Unlike IL-1, the maximal rates of production occurred sooner (2–4 hr); this finding likewise correlated to reported TNF mRNA levels, which decay after 4 hr [16].

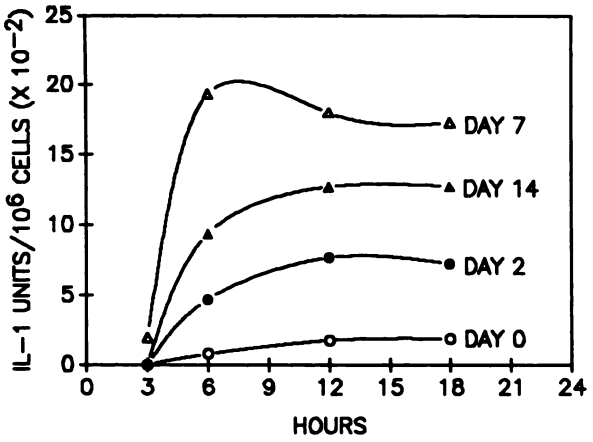

Fig. 4. Kinetics of interleukin 1 (IL-1) production by cultured peritoneal macrophages during the chronic exudative response. Macrophage cultures were prepared at the indicated intervals, and supernatants were collected at intervals over 18 hr following stimulation with 1 μg/LPS and then assayed for IL-1 activity. Points are mean of three determinations.

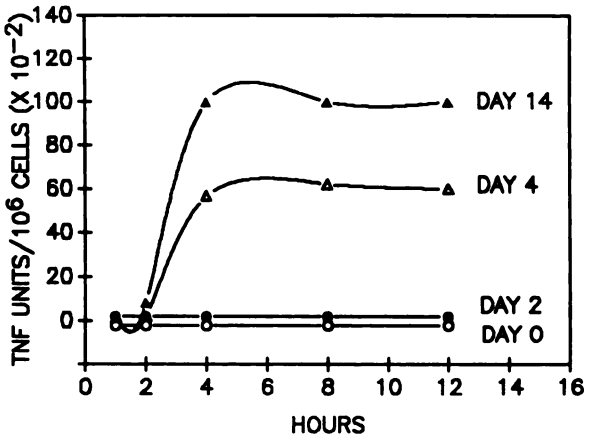

Fig. 5. Kinetics of tumor necrosis factor (TNF) production by cultured peritoneal macrophages during the chronic exudative response. Macrophage cultures were prepared at the indicated intervals, and supernatants were collected at intervals over 18 hr following stimulation with 1 μg/LPS and then assayed for TNF activity. Points are mean of three determinations.

Immunohistochemical Demonstration of Cell-Associated Monokine Expression During the Exudative Response

We have previously reported in situ monokine expression in cultured macrophages using antibodies specific for murine TNFα, IL-1α, and IL-1β [16,17]. Figure 6 shows the characteristic staining patterns observed for IL-1 and TNF. Both IL-1α and -1β displayed a diffuse cytoplasmic expression that accentuated the cell processes. In contrast, TNF was more localized in clumps and granules. Using this approach, we attempted to determine if changes in monokine production were related

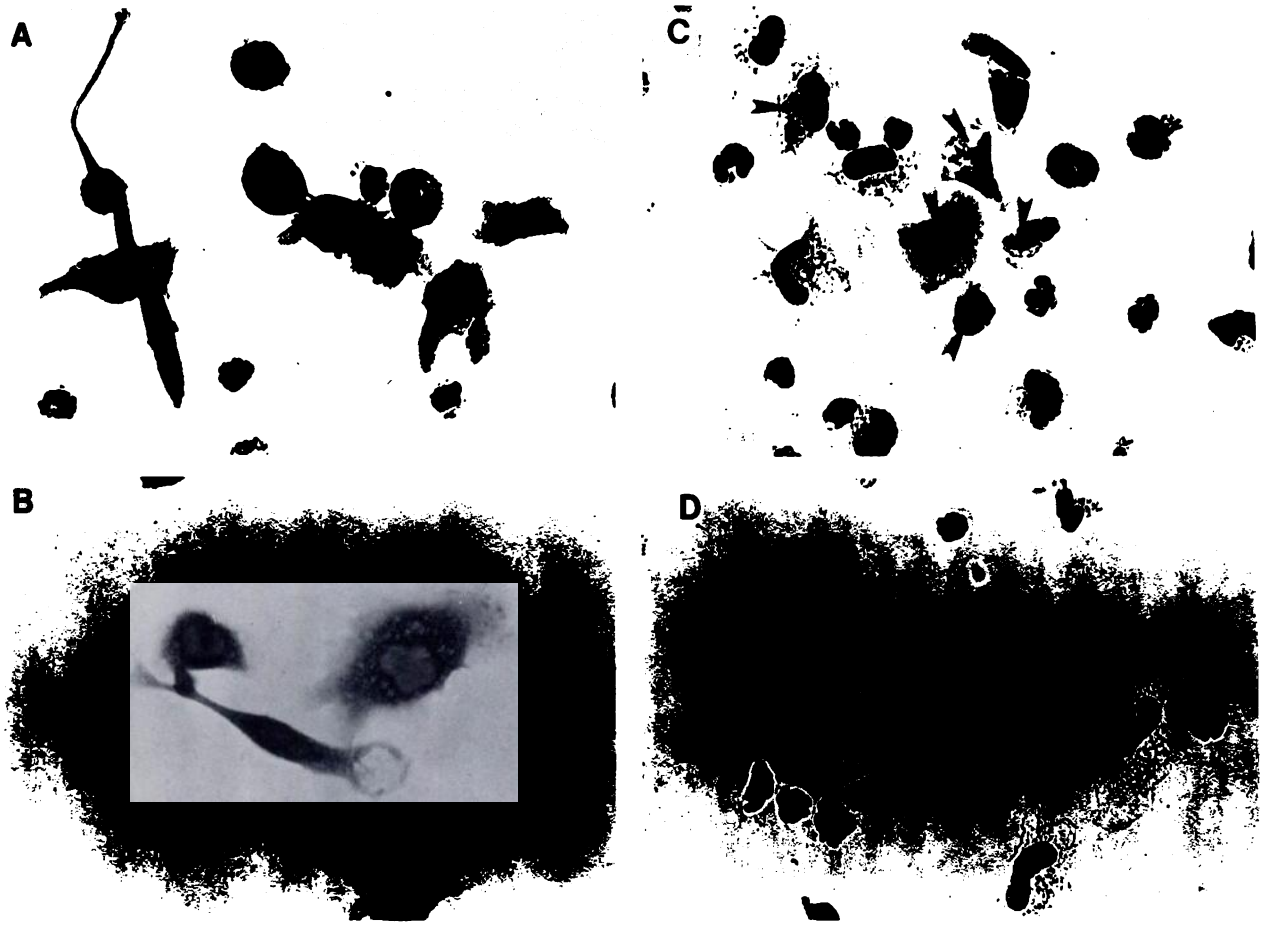

Fig. 6. Immunohistochemical staining patterns of monokines in cultured peritoneal macrophages. Macrophage monolayers were prepared from day 14 peritoneal exudates and stained for monokines using specific polyclonal antisera. A: IL-1α. B: IL-1β. C: TNF. D: Nonimmune serum control.

to changes in numbers of macrophages with cytoplasmic monokine expression.

Figure 7 shows the proportion of macrophages expressing cytoplasmic TNF during the course of the exudative response. Staining was performed 3 hr after LPS stimulus, the point previously shown to be the time of maximum expression [16]. Resident macrophages (day 0) showed no TNF expression consistent with the bioassay results. On day 2 after CFA injection, about 30% of macrophages showed detectable TNF synthesis following LPS stimulation. Thus cytoplasmic TNF expression was demonstrable while biologic activity was not (Fig. 2). This was probably due to the high degree of sensitivity of immunohistochemical detection compared with the more limited sensitivity of the bioassay. By days 7 and 14, the percentage of cells producing TNF had increased to about 60–65%, corresponding to the increased production of biologically active material.

Unlike TNF, a direct relationship between cytoplas-

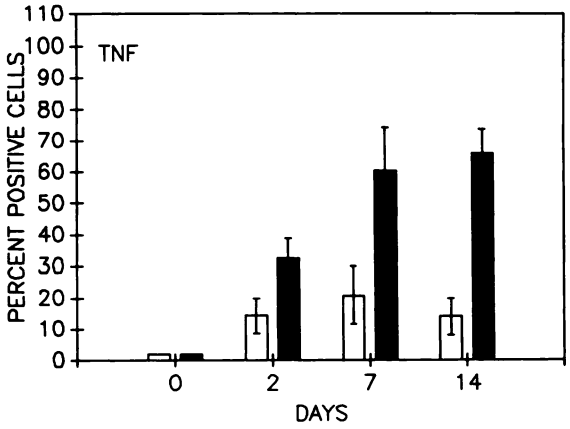

Fig. 7. Expression of cytoplasmic tumor necrosis factor (TNF) by cultured peritoneal macrophages during the chronic exudative response. Macrophage cultures were prepared at the indicated times following FCA injection and were stained for TNF 3 hr after LPS stimulation. Open bars, no LPS; filled bars, 1 μg/ml LPS. Bars are means ± SE of three determinations.

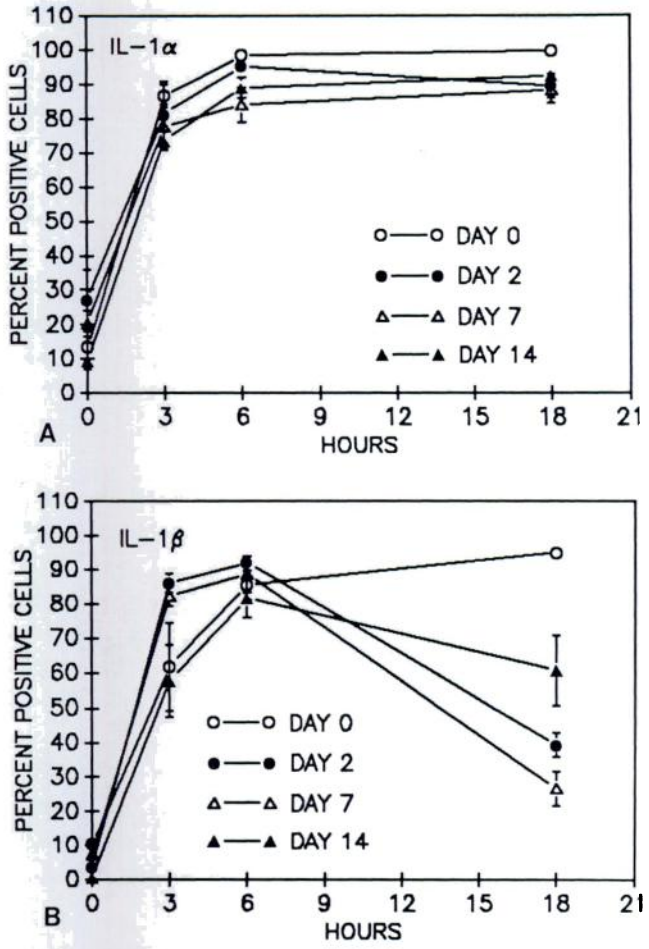

Fig. 8. Kinetics of interleukin 1α (IL-1α) (A) and IL-1β (B) cytoplasmic expression in peritoneal macrophages during the chronic exudative response. Macrophage cultures were prepared at the indicated times following FCA injection and were stained for IL-1α and IL-1β at time 0 and 3, 6, and 18 hrs after LPS stimulation. Points are mean ± SE of three to six determinations.

mic expression and secretion could not be established for IL-1. Figure 8 shows the kinetics of IL-1α and -1β expression in LPS-stimulated macrophages examined at intervals following CFA injection. Interestingly, IL-1α expression was similar among the cells over the 18 hr study period. Baseline expression was not significantly different among the populations. By 6 hr, nearly 100% of resident (day 0) and 85–95% of adjuvant-induced macrophages showed cytoplasmic IL-1α staining, which persisted until the end of the study period. IL-1β staining was likewise comparable over the first 6 hr, by which time 80–90% of cells showed positive staining. However, by 18 hr, there was decreased expression in day 2, 7, and 14 cells, whereas staining persisted in the day 0 cells. The decrease was most pronounced in the day 7 population. It should be noted that cytoplasmic IL-1α and -1β was induced in resident (day 0) cells with similar kinetics even in the absence of LPS, although secretion did not occur (data not shown). This was also observed in the other populations but to a much lesser degree. Hence resident cells appeared to have a lower stimulus threshold for cytoplasmic IL-1 expression.

Taken together, the immunohistochemical findings suggested that levels of TNF production could be related to the proportion of cells with cytoplasmic expression of this monokine. In contrast, IL-1α and -1β was expressed in nearly all macrophages. Hence differences in supernatant IL-1 levels must be related to the overall degree of synthesis/secretion and not to the proportion of cells with cytoplasmic IL-1 expression.

DISCUSSION

Based primarily on in vitro studies, numerous laboratories have described potential functional and often overlapping activities of IL-1 and TNF [3]. However, it has been difficult to establish their in vivo relevance and degree of participation in physiologic and immunopathologic mechanisms. Our laboratory has attempted to approach this problem by defining the sequence and degree of monokine production during inflammatory reactions and relating these findings to immunopathologic events [18]. With this information, reasonable in vivo studies can be designed in order to establish the physiologic significance of these molecules with regard to inflammation.

The present study provides strong circumstantial evidence that monokine production is orchestrated during chronic Freund's adjuvant-induced peritonitis. Specifically, there was a sequential appearance of IL-1 and TNF production, such that IL-1 production was maximal at 4–7 and TNF at 16–20 days after CFA injection. This agrees completely with our recently reported analysis of pulmonary granuloma macrophages isolated from synchronously developing granulomas [18]. In that model there was a clear sequence of early IL-1 production (2–4 days) followed by subsequent TNF production (8–16 days). There was likewise an association of IL-1 production with the recruitment phase of the lesion, whereas TNF production was maximal once the lesion was established. These studies suggest that monokine production is fitted to the phase of the inflammatory reaction.

It is reasonable to speculate that IL-1 is involved in the initial recruitment and activation of cells at sites of chronic inflammation. This would be consistent with some of its described in vitro functions such as induction of endothelial adhesion proteins [19], promotion of lymphocyte growth [20], and augmentation of lymphokine release [21]. TNF shares some overlapping functions with IL-1, but our data would suggest it may be more

important to the maintenance of the established reaction. It may sustain the reaction through induction of adherence proteins [19], activation of neutrophils [22], and regulation of fibrocyte growth and collagen synthesis [23,24].

Our findings showing the effect of cell density and LPS concentration on IL-1 and TNF production emphasize the importance of controlling these conditions when assessing monokine production. The observation that IL-1 was more sensitive to density changes than TNF production may reflect different mechanisms regulating production. This notion is supported by our recent study showing that the synthesis of these monokines is affected differently by prostaglandin E_2 [17].

The immunohistochemical studies further suggested profound differences in IL-1 and TNF synthesis and secretion. There were differences in patterns and numbers of cells staining. The proportion of cells with cytoplasmic TNF expression generally related to the observed levels of detectable bioactive material in the supernatant. However, despite significant differences in supernatant levels of bioactive IL-1, cytoplasmic expression of IL-1α and -1β was seen in 85–100% of cells from all sampled time points. Thus, while nearly all macrophage populations had the capacity for cytoplasmic IL-1 expression, they varied in their capacity to secrete or release it. Resident macrophages produced relatively little active IL-1, but by 4–7 days after exudate induction peritoneal macrophages showed profound IL-1 synthesis and secretion.

The mechanism of IL-1 secretion is presently unknown and is likely novel since the IL-1 precursor molecule lacks the characteristic leader sequence of secreted molecules [5,6]. It is likely that a molecular signal, possibly lymphokine, is required for maximum IL-1 synthesis. This notion is supported by in vitro studies showing augmented IL-1 synthesis following exposure to γ-interferon [25]. Matsushima et al. reported that IL-1 release may require the action of extracellular enzymes such as plasmin [26]. In view of their findings it would be of interest to attempt to correlate IL-1 secretion to levels of plasminogen activator, another macrophage product.

The only difference observed between IL-1α and -1β cytoplasmic expression was that the latter declined by 18 hr after LPS stimulus in adjuvant-elicited macrophages. This may indicate transport of IL-1β from the cytoplasm since there is mounting evidence that IL-1β is the predominant secreted form [7,27]. Using solid phase immunoabsorbents, we have noted IL-1β to be at three to fivefold higher levels than IL-1α in macrophage supernatants (manuscript submitted). Our findings may also reflect different mechanisms regulating the production of IL-1α and -1β, resulting in the selective down-regulation of one but not the other.

A caveat should be mentioned regarding immunohistochemical localization. This method is highly sensitive but qualitative. No information concerning quantities of target protein per cell is offered; therefore subtle differences in synthesis are overlooked. With eventual application of more sophisticated image analysis, quantitation can be performed and will likely provide a more accurate definition of the changes in levels of cytoplasmic monokines.

In conclusion, our study shows sequential changes in IL-1 and TNF expression and secretion by macrophages recruited during the chronic exudative response. These findings suggest that monokine production may be fitted to specific stages of the reaction. They also imply a complex system of signals that govern the sequential appearance and secretion of monokines.

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