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Influence of Different Conditions on Kinetics of Tumor Necrosis Factor Alpha Release by Peripheral Blood Mononuclear Cells after Stimulation with *Cryptococcus neoformans*: a Possible Explanation for Different Results

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In available literature, different kinetics for tumor necrosis factor alpha (TNF-α) release by peripheral blood mononuclear cells are reported upon stimulation with *Cryptococcus neoformans*. Results in this study showed that shaking cells gives faster kinetics of TNF-α release while large working volumes give lower TNF-α concentrations. Different experimental conditions thus influence kinetics of TNF-α release by phagocytes.

Studies with human cells have suggested a critical role for natural effector cells such as mononuclear cells in the host defense against *Cryptococcus neoformans* infections (7, 9). Subsequent studies have demonstrated that mononuclear cells release tumor necrosis factor alpha (TNF-α) among other cytokines in response to *C. neoformans* (3, 8). TNF-α has diverse immunoregulatory, metabolic, and inflammatory activities, among which are the induction of cell adhesion molecules (resulting in phagocyte accumulation) and the stimulation of production of other cytokines (2, 6, 12). Its autocrine role in the production of other cytokines makes it a pivotal cytokine in initiating and amplifying immune responses. TNF-α is a cytokine known to influence the outcome of cryptococcal infections (4). While there is consensus among in vitro studies on the fact that TNF-α is released by phagocytes after cryptococcal stimulation, there appear to be differences in time kinetics of release of this cytokine. In our recently published report (3), together with the reviewers of the paper, we were puzzled by the differences between studies (3, 5, 8, 11) in concentrations and kinetics of TNF-α release by peripheral blood mononuclear cells (PBMC) when stimulated by *C. neoformans*. The following study investigated the influence of different experimental conditions on the kinetics of release of TNF-α by PBMC on stimulation by *C. neoformans*.

C. neoformans NIH 37 was used in these experiments. NIH 37 is a thinly encapsulated strain (<0.05-μm capsule diameter). Cells were prepared and killed as described previously (3). Cells were opsonized with 10% normal human serum for 30 min at 37°C and adjusted to required concentrations. Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 (Sigma Chemical Co., St. Louis, Mo.) was used as a positive control. PBMC were isolated as described previously (1), and the concentrations were adjusted as required. PBMC were stimulated either with *C. neoformans* in the presence of 10 μg of polymyxin B sulfate ml^{-1} (Sigma) or with LPS (1 μg ml^{-1}) at indicated concentrations and volumes. Stimulations were carried out in either polystyrene round-bottomed tubes (Becton Dickinson, Mountain View, Calif.) or 96-well polypropylene U-bottomed plates (Costar, Cambridge, Mass.). The cells in the tubes were either kept stationary or shaken continuously at 300 rpm. Incubations were done at 37°C, and supernatants were harvested after 3 and 18 h of stimulation. TNF-α production was determined by an enzyme-linked immunosorbent assay as described previously (3). Statistical differences were analyzed by the paired Student *t* test. $P < 0.05$ was considered significant.

Previous observations by our group have shown that the induction of TNF-α release by *C. neoformans* is dose dependent, with higher fungus ratios inducing more TNF-α release in PBMC (3). Levitz et al. (8) reported similar findings, observing a fourfold increase in TNF-α levels when the fungus/cell ratio was raised from 1:1 to 10:1. The main difference between our observations and those of Levitz et al. is that the latter reported near maximal levels of TNF-α at 18 h (fungus/cell ratio, 10:1), while in our circumstances TNF-α levels dropped to background levels after 18 h of stimulation. One major difference between our experimental setup and that of Levitz et al. is that we performed our stimulations in tubes that were continuously shaken throughout the course of the experiment while Levitz et al. performed their stimulations in stationary 96-well plates.

Using the fungus/cell ratio of 10:1, we investigated what the influence of shaking might be on kinetics of TNF-α release induced by *C. neoformans*. Shaking resulted in very rapid release of TNF-α, and a fast decline in levels ensued for both *C. neoformans*- and LPS-stimulated PBMC (Fig. 1). A similar response was seen for gamma interferon release by PBMC stimulated with *C. neoformans* (2a). The reduction in levels of the two cytokines might relate to their being broken down to fragments that are not detectable by the enzyme-linked immunosorbent assay. Meanwhile, when tubes were left standing, kinetics of TNF-α release appeared to be protracted. At 3 h, detectable levels of TNF-α were seen and were still rising at 18 h. Similar kinetics were seen in 96-well plates (Fig. 1). The levels of TNF-α at 18 h in the stationary tubes and 96-well plates were significantly higher ($P < 0.05$) than those obtained at 18 h in the supernatants of tubes kept shaken throughout the experiment. The kinetics of LPS-induced TNF-α release closely resembled the patterns seen with *C. neoformans*.

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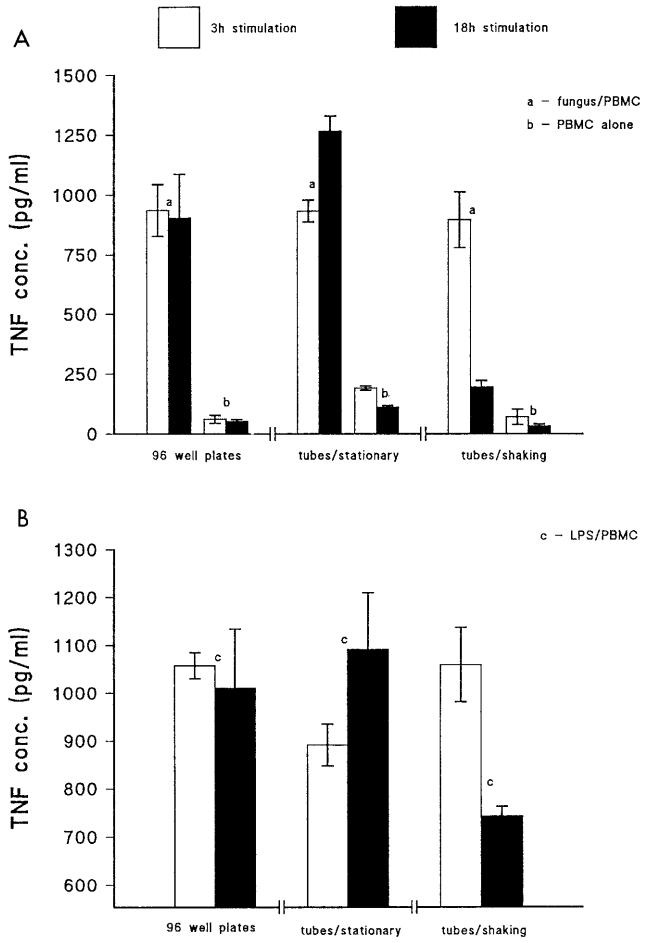

FIG. 1. A total of 10^6 PBMC were stimulated with either 10^7 *C. neoformans* cells or 1 μg of LPS (*E. coli* O111:B4) ml^{-1} in a final volume of 200 μl. Cells were kept stationary in either polystyrene tubes or 96-well polypropylene plates. A set of tubes was continuously shaken during stimulation. (A) Time kinetics of TNF-α release by PBMC when *C. neoformans* is the stimulus; (B) kinetics when LPS is the stimulus. In both panels, faster kinetics of TNF-α release are seen in the tubes kept shaken, with levels dropping after 18 h. High levels of TNF-α still remain in stationary tubes and 96-well plates after 18 h. PBMC incubated with buffer show basal levels of TNF-α release for all three conditions. Results are means ± standard errors of the means of three experiments performed with three different donors.

We next investigated the influence of volume in which a fixed concentration of cells were suspended on the kinetics of TNF-α release. Our observations were that the smaller the volume, the higher the concentrations of TNF-α detected per volume (Fig. 2). Significant differences ($P < 0.05$) were seen between 0.1 and 2 ml. Large volumes altered the time kinetics of release such that at 18 h levels were still rising (Fig. 2). This observation was most apparent with 2 ml. It is noteworthy that the group of Vecchiarelli et al. (11) worked with a fungus/cell ratio of 1:1 in 24-well plates, presumably with large volumes, and they reported an even more protracted process. They observed low levels of TNF-α at 3 h, near maximal levels at 18 h, and levels continuing to rise at 48 h. The low ratios and large volumes might therefore have a bearing on these extended time kinetics as we showed in our investigations. We also reported recently that cryptococcal cell wall components induce TNF-α release by PBMC (3). We employed our usual conditions of shaking cells (final volume, 200 μl) in tubes. We observed kinetics of TNF-α release similar to those seen with whole *C. neoformans*. Delfino et al. (5) also tested the cryptococcal components for their TNF-α-inducing abilities in whole blood and separated PBMC. They worked in 1-ml volumes and reported a slow decline in TNF-α levels after 18 h of stimulation.

One possible explanation for the difference in time kinetics observed between the different conditions might be the contact between effector and target cells. Shaking increases the probability of contact between fungus or its components and PBMC, resulting in a faster induction of TNF-α release. Similarly, shaking increases contact between soluble LPSs that have a micelle structure (10) and PBMC. On the other hand, when tubes and/or plates are left standing or when large working volumes are used, the chance of contact between cells is reduced, resulting in slower kinetics of TNF-α release. Other possible explanations for the observed results for the effects of

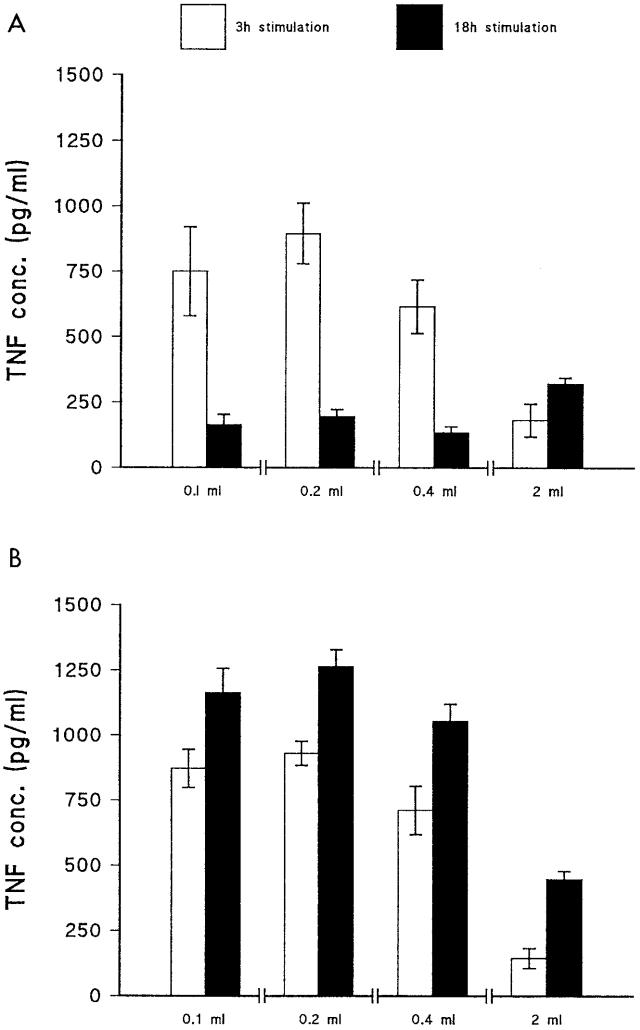

FIG. 2. A total of 10^6 PBMC were stimulated with 10^7 *C. neoformans* cells, and the volume of buffer in which cells were suspended varied. (A) Tubes were shaken continuously through 3 and 18 h of stimulation; (B) tubes were kept stationary. Optimal levels of TNF-α release were seen with the 200-μl volume. Increasing the volume resulted in lower concentrations in both panels. Kinetics of TNF-α release similar to those in Fig. 1 were seen with volumes up to 400 μl. Larger volumes, i.e., 2 ml, resulted in changed kinetics of release under conditions in the experiments, with levels still rising at 18 h. Basal levels of TNF-α were seen in control PBMC. Results are means ± standard errors of the means of three experiments performed with three different donors.

volume might relate to the supplements in the medium in which the cells were kept.

We conclude that different experimental conditions influence the time kinetics of TNF-α release by PBMC and possibly other phagocytes when stimulated by *C. neoformans*. These factors should be taken into consideration when making comparisons between studies by different groups, particularly in the determination of concentrations. The kinetics of release of a pivotal cytokine like TNF-α in immune responses will reflect on the course of an infection, its containment, and ultimately its elimination by phagocytes. Future studies will be required to ascertain the cytokine profile that is actually present during cryptococcal infections in humans and mice.

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