Vol. 141, No. 2, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 15, 1986 Pages 818-824

Tumor Necrosis Factor-Induced Alterations in Circulating Leukocyte Populations

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Received October 31, 1986

Tumor necrosis factor is a potent agent possessing diverse biological functions. We investigated the effects of intravenous administration of human recombinant tumor necrosis factor (TNF) on immune cell populations in CBA/J mice. The animals developed a significant lymphopenia and neutrophilia both reaching a maximum at 4 hours post-injection with a trend towards resolution to normal values by 6 hours. The lymphopenia was both relative and absolute. Similarly, the neutrophilia was both relative and absolute and was due to the presence of both immature and mature neutrophils. As the neutrophilia and lymphopenia occurred concomitantly, there was no difference at any time point in the total number of peripheral blood white cells. Extensive controls were done to rule out LPS contamination in the TNF preparation. These data demonstrate the potent effects of intravenous administration of human recombinant tumor necrosis factor on peripheral blood constituents. © 1986 Academic Press, Inc.

Tumor necrosis factor (TNF) is a macrophage derived peptide that has a number of functions directed against diverse cell types (1). Several *in vitro* studies have demonstrated TNF's ability to modulate the proliferation of mammalian cell lines (2), increase endothelial adhesiveness (3) and enhance phagocytic cell activity (4). *In vivo* studies have generally focused on the oncolytic activity of the TNF on solid tumors and characterization of physiological properties in various species has been limited.

Recent work by Beutler (5) has suggested that TNF represents the final mediator molecule that accounts for the changes observed when lipopolysaccharide (LPS) is given systemically. While previous investigations have demonstrated that the intravenous administration of LPS

Abbreviations: LPS-lipopolysaccharide, PMN-polymorphonuclear neutrophils, TNF-tumor necrosis factor

0006-291X/86 $1.50
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causes marked changes in peripheral blood leukocyte populations when given to human (6-8) or calves (9), we now report similar effects induced by the systemic administration of human recombinant TNF. Kinetic studies showed alterations in peripheral blood leukocyte populations that began within 15 minutes and persisted to 6 hours. Numerous control studies showed that these changes were not due to lipopolysaccharide contamination.

Materials and Methods

Animals: Female CBA/J mice (Jackson Labs, Bar Harbor, ME were used throughout this study. The mice were maintained under standard care and given food and water *ad libitum*.

In vivo effects of TNF: Recombinant human TNF (A gift from Dr. Leo Ling, Cetus, Corp., Emeryville, CA) was administered to mice via a single 200 µl bolus tail vein injection. Prior to use, the TNF stock solution was diluted with pyrogen-free normal saline 1 to 20 such that 1 µg/mouse was administered in 200 µl (1 µg TNF equals 10^4 units of activity). Control mice received 200 µl of normal saline and were sacrificed 2 hours later.

Kinetics study: At specific time points post-administration, the animals were sacrificed and blood was anticoagulated with 0.1 ml of 50 mM EDTA (Sigma, St. Louis, MO), 10 mM phosphate-buffered saline pH 7.4. The volume was measured and a white count performed with a unopette (Becton-Dickinson, Rutherford, NJ). A smear was prepared, stained with Wright-Geimsa and a 100-cell differential was done with the observer blinded as to the nature of the specimen. Immature PMNs were defined as those cells with plump, ring nuclei (10). Time 0 represents values from normal mice.

Controls: As determined by the limulus assay the LPS contamination was less than 38 ng/mg of protein, an amount corresponding to a less than 1.5 ng LPS/mouse. LPS (Sigma Chemical Co. St. Louis, MO) was injected in a 200 µl bolus intravenously at 1.0 ng/mouse and the blood was collected 2 hours later. A second control consisted of inactivating LPS activity by mixing the diluted TNF preparation with polymyxin B (Sigma Chemical Co., St. Louis, MO) for 30 minutes at 37°C (11). The third control consisted of heat inactivation of the TNF preparation. Controls were compared to treated mice by Students t-test for all studies.

Results

Human recombinant TNF had a dramatic effect on the peripheral blood leukocytes. In a normal CBA/J mouse the predominant peripheral blood leukocyte is the lymphocyte, representing 77 ± 10% of cells, while neutrophils comprise 21 ± 10% (mean ± SD). Injection of normal saline causes some alterations compared to normal mice, but not as dramatic as with the TNF challenge. Two hours post-injection of 1 µg TNF, lymphocyte numbers fell by more than 64%. In contrast, there was a 250% increase in the percentage of peripheral blood neutrophils. These alterations in the

Table 1

Experiments controlling for lipopolysaccharide (LPS) contamination of the TNF preparation

Material Injected	% Cells		Cells/ml ($x10^4$)		
	Lymphs	PMNs	Total	Lymphs	PMNs
Nothing	77±10	21±10	374±154	294±135	75±37
Normal Saline	58±9	41±9	346±10	148±55	115±65
TNF 1μg	28±6*	70±6*	300±68	77±29*	193±80*
TNF 1μg, Heated	58±12	41±12	427±127	241±3	185±97
LPS 1ng	55±9	45±9	355±157	200±59	180±97
TNF 1μg + Polymyxin B	43±1*	57±1*	310±60	134±27	176±34

The material was injected intravenously and the blood collected 2 hours later. The TNF preparation caused a lymphopenia and neutrophilia which was both relative and absolute. Heating the TNF abolished this activity. LPS did not affect the peripheral blood leukocyte populations. Mixing the TNF with polymyxin B, to inactivate any possible LSP contamination, did not eliminate the preparation's ability to induce alter leukocyte populations. Each point is the mean ± SD for 3 to 8 mice. * = $p<.05$.

circulating white blood cell populations were not only relative, but were also absolute, as indicated by the changes in the total numbers of lymphocytes and neutrophils. The total white count did not vary significantly since the increase in PMNs offset the decrease in lymphocytes. These results are summarized in Table 1.

Lipopolysaccharide (LPS) contamination can induce profound changes in the peripheral blood values (6-8), therefore, it was important to rule out any effects due to LPS contamination. Three separate control experiments were conducted to document that the observed effects were due to the TNF preparation and not LPS contamination. First, LPS was not detectable in the TNF sample by the limulus assay, and an intravenous challenge of LPS at the levels potentially found in the TNF preparation did not affect our system. Second, heating the TNF preparation, which destroys TNF activity but not LPS, eliminated the observed effects of the TNF. Finally, mixing the TNF with polymyxin B, which has been reported to bind and reduce the biologic activity of LPS did not abolish the activity of the TNF (11). These results are also summarized in Table 1.

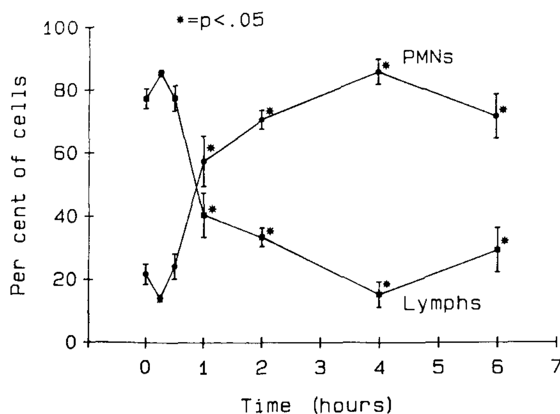

Figure 1. Kinetics of IV administration of 1.0 μg TNF in CBA/J mice. Peripheral blood was collected at the indicated time intervals and a differential count performed. A brief relative lymphocytosis and neutropenia was followed by a more pronounced and longer lasting lymphopenia and neutrophilia. Each value is mean ± SEM for 3 to 14 mice * = $p<.05$ compared to normal mice (Time 0).

The kinetics of the TNF-induced changes are shown in Figure 1. Upon intravenous infusion of TNF, there was a brief lymphocytosis at 15 and 30 minutes followed by a dramatic reduction in the percent of circulating lymphocytes at 1 hour. This decrease in the per cent of circulating lymphocytes persisted up to the 6 hour point where there was still a 30% reduction. The change in the circulating lymphocytes was mirrored by alterations in the peripheral PMN population. There was a sharp reduction in the per cent of circulating PMNs at 15 and 30 minutes followed by an increase at 1 hour. As with the lymphopenia, the neutrophilia persisted throughout the 6 hour study period. These relative changes in lymphocytes and PMNs were matched by absolute changes in the cell numbers. The kinetics of the absolute numbers of lymphocytes and PMNs post-TNF challenge are shown in Figure 2. Lymphocyte numbers demonstrated a gradual, but steady, decline reaching a nadir at 2-4 hours post-TNF injection. PMN numbers appeared to increase in a step-wise fashion, reaching the first plateau at 1-2 hours post-challenge and a second plateau at the 4-6 hour mark. To further elucidate the composition of the neutrophilia, we enumerated the immature neutrophils, since the peripheral neutrophilia may have been due to entry of marginated PMNs or

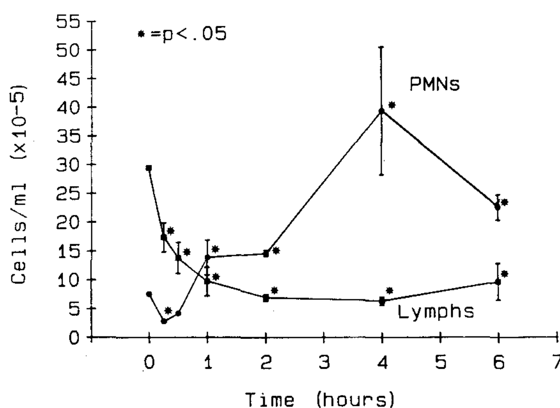

Figure 2. Kinetics of total cells following IV administration of 1.0 μg TNF. At the indicated time points the blood was collected and a white count done. A brief absolute neutrophilia was followed by a more pronounced and longer lasting neutrophilia, while the lymphopenia became evident even at the earliest time point (15 minutes). Each value is mean ± SEM for 3 to 14 mice * = p<.05 compared to normal mice (Time 0).

the recruitment of immature cells from the bone marrow. Immature PMNs were identified morphologically as peripheral blood neutrophils with ringed nuclei (10). As shown in Figure 3, the augmentation in the circulating PMN population in response to TNF challenge was primarily due to recruited immature neutrophils, although the number of mature cells was also significantly increased over control values at 4 and 6 hours post-challenge. The recruitment of immature PMNs occurred earlier, as soon as 1 hour, peaked at 2-4 hours, and persisted up to the end of the study period.

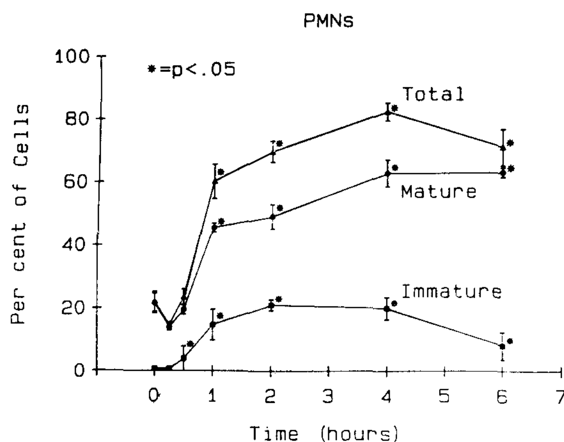

Figure 3. Kinetics of immature neutrophils. A differential count of peripheral blood smears showed increasing numbers of immature neutrophils. The neutrophilia was due to both mature and immature forms. Each value is mean ± SEM for 3 to 14 mice * = p<.05 compared to normal mice (Time 0).

Discussion

The above study demonstrates that human recombinant tumor necrosis factor (TNF) administered intravenously in CBA/J mice induces profound alterations in the peripheral blood leukocytes. These changes consisted of an early mild lymphocytosis followed by a dramatic lymphopenia, and a parallel neutropenia followed by neutrophilia. These changes were found to be both relative and absolute. The neutrophilia was due predominantly to immature cells. Control studies demonstrated that potential LPS contamination was not contributing to our results.

It has been postulated that many of the biologic effects of LPS are mediated through TNF; that is LPS induces TNF secretion by mononuclear phagocytes and the latter mediator causes the *in vivo* alterations. Evidence for this hypothesis is provided by studies that show a rapid rise in TNF titers in the peripheral blood levels of infected mice challenged with LPS (12). Further support is found in Beutler's work where antibodies directed against murine TNF was able to prolong survival in mice challenged with a lethal dose of LPS (5). It is also interesting to note that the TNF-induced peripheral blood alterations that we observed in our investigation are very similar to those induced in humans (6-8) and calves (9) following intravenous infusion of LPS. In these previous studies, an early neutropenia was followed by a marked neutrophilia, due primarily to immature PMNs, while the lymphocyte populations were reduced following LPS challenge. Since we are able to induce similar changes by administering TNF these studies also suggest that the LPS may be acting through release of TNF. Further studies are underway to elucidate the mechanism of TNF's effect on the lymphocytes.

Several questions are raised by this study. Although the lymphocytes are reduced in both number and per cent, the effects of TNF on specific lymphocyte populations is not clear. It is not known if particular subsets were diminished or if there was an effect on all lymphocyte populations. Previous work demonstrated that TNF was cytotoxic to normal

murine B cells (13); thus, the reduction of lymphocytes in the peripheral blood may have been through a direct cytotoxic action. Studies are underway to characterize the phenotypes of the peripheral blood lymphocytes to ascertain if the B cells are preferential targets. In addition, TNF may cause changes by altering the vascular endothelium. This is especially intriguing since Pohlman (14) and Pober (15) have demonstrated an up-regulation of a specific endothelial cell surface protein by TNF that may be involved in leukocyte adherence.

These studies further support the hypothesis that TNF may serve as an important peptide mediator with regard to *in vivo* LPS-induced alterations.

References

1. Old, L.J. (1985) Science 230, 630-632.
2. Sugarman, B.J., Aggarwal, B.B., Hass, P.E., Figari, I.S., Palladino, M.A. and Shepard, H.M. (1985) Science 230, 943-945.
3. Yu, C., Haskard, D., Cavender, D., and Ziff, M. (1986) J. Immunol. 136, 569-573.
4. Shalaby, M.R., Aggrawal, B.B., Rinderknecht, E., Svedersky, L.P., Finkle, B.S. and Palladino, M.A. (1985) J. Immunol. 135, 2069-2073.
5. Beutler, B., Misark, I.W., and Cerami, A.C. (1985) Science 229, 869-871.
6. Mechanic, R.C., Frei, E., Landy, M., and Smith, W.W. (1962) J. Clin. Invest. 41, 162-172.
7. Gilbert, H.S., Rayfield, E.J., Smith Jr., H., and Keush, G.T. (1978) Metabolism 27, 889-899.
8. Gale, R.P., Opelz, G., and Golde, D.W. (1977). Br. J. Haem. 36, 49-57.
9. Deldar, A., Naylor, J.M., and Bloom, J.C. (1984) Am. J. Vet. Res. 45, 670-677.
10. Schermer, S., (1967) In, The Blood Morphology of Animals. F.A. Davis, Co. Philadelphia.
11. Neter, E., Gorzynski, E.A., Westphal, O. and Luderitz, O. (1958) J. Immunol. 80, 66-72.
12. Ha, D.K.K., Leung, S.W., Fung, K.P., Choy, Y.M., and Lee, C.Y. (1985). Int. J. Immunopharmac. 7, 1-6.
13. Playfair, J.H.L., de Souza, J.B., and Taverne, J., (1982) Clin. Exp. Immunol. 47, 753-755.
14. Pohlman, T.H., Stanness, K.A., Beaty, P.G., Ochs, H.D. and Harlon, J.M. (1986) J. Immunol. 130, 4548-4553.
15. Pober, J.S., Bevilacqua, M.P., Mendrick, D.L., Lapierre, L.A., Fiers, W., and Gimbrone, M.A. (1986) J. Immunol. 136, 1680-1687.