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# The altered activation state of macrophages isolated from ascitic fluid of patients with peritoneal carcinomatosis

## ABSTRACT

***Background:*** *Cells of the monocyte/macrophage (Mo/Mph) lineage are capable of efficient killing of human nucleated tumor cells and this activity represents an important mechanism of host anti-tumor defense. However, in many cases developing neoplasms appear to be capable of impairing steps in this complex mechanisms as a means of avoiding immune destruction. The aim of this study was to investigate functional properties (phagocytosis and nitric oxide production) of cancer patient's mononuclear phagocytes*

***Materials and methods:*** *Mononuclear phagocytes used in this study were obtained following the plastic adherence technique. Nitric oxide production, measured by the Griees reaction, and phagocytosis test were used to quantify the monocyte/macrophage functions in 10 patients with peritoneal carcinomatosis*

***Results:*** *Our result showed that phagocytic activity of peripheral blood monocytes (PBMo) was not altered, whereas tumor-associated macrophages (TAM) fromascitic fluid had impaired phagocytic activity. In addition, crude supernatant from short-term cultures of the peritoneal cells obtained from a cancer patient with peritoneal carcinomatosis have inhibitory effect on Mo/Mph phagocyte function, suggesting that tumor derived soluble factors are responsible for TAM inhibition. Moreover, TAM displayed decreased capacity to produce and/or release nitric oxide (NO), whereas monocytes produce NO at levels comparable to those of corresponding controls.*

***Conclusion:*** *Taken together, these data suggest that in the mechanisms of tumor dependent down-regulation of host defense functional disability of TAM may play an important role.*

***Key words:*** *Monocytes; Macrophages-peritoneal; Nitric-oxide; Neoplasms; Phagocytosis*

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## INTRODUCTION

The mononuclear phagocyte system is a dynamic network of functionally heterogeneous cells of the monocyte/macrophage lineage that is critical to the integrity of the host defense. Mononuclear phagocytes express complex functions that relate to immunity, inflammation and thrombosis (1). Hence, it is not surprising that they have an important role in the immunobiology of neoplastic tissues. The presence of macrophages within solid tumors has been documented for over 100 years. However, some functional aspects of these cells remain a mystery. For example, cells of the monocyte/macrophage lineage, when appropriately activated, can kill some tumor cells directly or can elicit tumor-destructive reactions, and yet they support the growth of other tumors. In vitro, Mph participates in tumor cell killing through varied processes that can involve the release of soluble factors (i.e., tumor necrosis factor-a (2,3), lysosomal enzymes (4,5), oxygen radicals (5-8) and nitric oxide (9-12)), the activation of antibody-dependent cell-mediated cytotoxicity, antigen presentation to T cells and phagocytosis (13).

Although tumor-associated macrophages have been extensively studied, only within the last few years has the ability of neoplastic cell phagocytosis by TAM gained increasing attention. However, earlier studies have demonstrated an increase in the phagocytic activity of TAM from tumor bearing mice (14,15). More-recent work showed positive correlation between neoplastic cells phagocytosis and prognosis (16,17). In this regard, Fiumara et al. 1997, showed that distant metastases developed in none of tumors with evident neoplastic cells phagocytosis (18). Thus, immune reaction involving neoplastic cell phagocytosis by TAM, may play a role in development of distant metastases.

Nitric oxide is also a powerful weapon in macrophage-mediated host defense. Several agents, including cytokines, microbial products and tumor cells, can stimulate mouse macrophages for NO release (19,20). In contrast to mouse macrophages, human mononuclear phagocytes stimulated with cytokines or lipopolysaccharide failed to produce NO (21,22). However, it has only recently been appreciated that to produce this mediator, human macrophages must be mature (cultured in vitro 7-10 days) and must be activated through the crosslinking of a variety of cell surface receptors (23-26).

The present study was undertaken to investigate the functional properties of cancer patients mononuclear phagocytes isolated from different anatomical sites.

*Abbrevations: TAM - tumor associated macrophages; PBMo - peripheral blood monocytes; Mo - monocytes; PEM - peritoneal macrophages; Mph - macrophages; NO - nitric oxide; AF - ascitic fluid; PL - plasma; PI - phagocytic index; PP - percent of phagocytosis; API - absolute phagocytic index*

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## MATERIALS AND METHODS

*Patients.* We analyzed a group of 10 patients with peritoneal carcinomatosis (6 patients with primary breast cancer and 4 patients with primary ovarian cancer), none of whom had received any chemotherapy or endocrine therapy during the last three months. Control group consisted of 10 healthy individuals and 10 stable patients on continuous ambulatory peritoneal dialysis. Abnormal peritoneal situations were excluded by blood cell count, nocturnal peritoneal effluent cell population and bacterial culture.

*Preparation of monocytes.* Mononuclear cells were isolated from heparinised venous blood using a density gradient (Lymphoprep 1.077, NYCOMED PHARMA AS, Oslo, Norway). Adherent monocyte monolayers were obtained by plating the mononuclear cells in 10cm plastic dishes for 2h at 37°C in 5% $C0_2$ . The adherent population after 2h incubation was >90% nonspecific esterase positive (Sigma Chemical Co). After the adherence, Mo to be used were placed on ice for 30 min. in 0.2% EDTA (ZORKA, Šabac, YU), gently scraped free of the plate, and resuspended in supplemented media: RPMI 1640 (GIBCO Europe) contained 5% heat inactivated autologous AB serum, 100U/ml of penicillin (ICN Galenika, Zemun, Yu), 100mg/ml streptomycin (ICN Galenika, Zemun, Yu) and 2mM L-glutamine (Merck, Germany). Viability remained >95% by acridine orange/etidium bromide exclusion.

*Preparation of peritoneal macrophages.* Peritoneal macrophages (PEM) were isolated from the nocturnal peritoneal effluent after an overnight dwell time. The complete volume collected was centrifuged (450g for 20min.) and cells were pooled. The adherent population was obtained following the plastic-adherence technique described by Kennedy (27). This procedure provides a population consisting of greater than 95% Mph as determined by nonspecific esterase staining. Viability of cells was >95%. Peritoneal Mph was cultured in supplemented media RPMI 1640.

*Preparation of TAM.* TAM was isolated from ascitic fluid (AF) of cancer patients with peritoneal carcinomatosis. Collected volume of ascitic fluid was centrifuged (450g for 20 min.) and cell pellet was resuspended in supplemented media RPMI 1640. The resulting suspension was then spun through Lymphoprep density gradient (1.077) to remove dead cells, erythrocytes and debris. The viable cells were washed twice and the adherent population consisting of 90% nonspecific esterase positive cells was obtained after 2h incubation at 37°C in 5% $C0_2$. After adherence TAM were placed on ice for 30min. in 0.2% EDTA, gently scraped free of the plate and resuspended in supplemented RPMI 1640.

*Phagocytosis assay.* We used an assay developed by Vujanović and Arsenijević with minor modification (28). Isolated mononuclear phagocytes (Mo, PEM and TAM) were suspended in medium Haemacel (Jugoremedia, Zrenjanin, Yu) at a concentration of 1 x $10^6$/cells/wial in a volume of 400ml. The heat inactivated yeast particles, labelled with Neutral red (Merck, Germany) were then added at a 1:12 E:T ratio and cells were centrifuged at room temperature for 5min at 50g. Mixed suspension was incubated for 1h at 37°C. Noningested yeast particles was removed by washing twice with ice cold 0.02% EDTA. At least 300 cells were assessed per well, and each experiment used duplicate sample wells per condition. The average number of yeast particles ingested per one cell was defined as the phagocytic index (PI), whereas the percentage of cells ingesting at least one yeast particle was defined as the percent of phagocytosis (PP). Absolute phagocytic index (API) represents a number of yeast particles ingested per 100 cells (API = PP x PI).

*Nitrite determination.* Isolated mononuclear phagocytes were adhered in 96 well flat-bottom plates (Spectra, Čačak, Yu) at 0.2 x $10^6$ cells/well. Cells were then cultured for 24h at 37°C in 5% C02 in supplemented media RPMI 1640. Nitrite concentrations in cell free supernatants were measured by the Griees reaction according to the protocol of Stuehr and Nathan (29), and serve as a reflection of NO production. Nitrite concentrations in plasma (PL) and ascitic fluid (AF) were determined by Griess reaction following a procedure described by Green (30).

*Immunomodulation.* Mononuclear phagocytes (Mo and PEM) from control individuals were pretreated for 1h with different concentrations (1:10; 1:$10^3$; 1:$10^4$;) of crude supernatants from short-term cultures of the peritoneal cells obtained from patients with peritoneal carcinomatosis, and phagocytosis assay was performed.

The obtained results were analyzed using two-factorial analysis of variances in SPSS for Windows PC programs. The study was permitted by the Ethic Committee of Medical School in Kragujevac.

## RESULTS

*Blood cell count.* The total and differential count of circulating leukocytes were determined from a sample of the heparinised venous blood. We found that cancer patients showed normal count of total leukocytes, but the increase number of granulocytes (neutrophilia) accompanied by the decreased number of lymphocytes (lymphocytopenia). The granulocytes/lymphocytes ratio was significantly higher in cancer patients than in normal controls. The mean number of

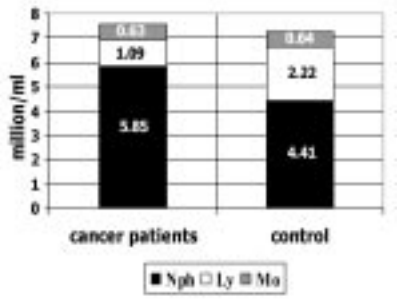


**Figure 1.** The total and differential cell count. Cancer patients showed an increased number of lymphocytes (Ly), and neutrophils (Nph), but normal counts of monocytes (Mo) and total leukocytes. Data are expressed as the mean of ten different experiments

monocytes was not significantly different between the cancer patients and control group (Figure 1).

Mononuclear cell phagocytosis. Phagocyte function of PBMo and TAM were synchronously examined in 10 cancer patients. Comparison between patients and their corresponding normal controls did not reveal any difference in phagocytic activity of PBMo. Hence, PP (Figure 2), PI and API were not significantly different between investigated groups. In contrast, TAM showed a marked impairment of phagocytic

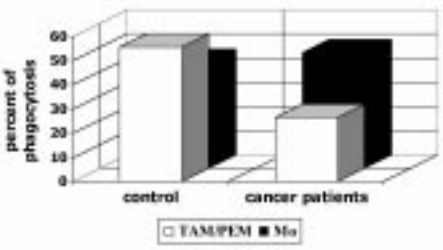


**Figure 2.** Phagocytosis assay. Mononuclear phagocytes (1x$10^6$) from cancer patients (Mo and TAM) and controls (Mo and PEM) were incubated for 1h with Neutral red labelled heat inactivated yeast particles. Effector: target ratio was 1:12. Percent of phagocytosis was determined using a light microscope. Values represent the mean of duplicate samples from ten experiments

activity. The significant inhibition of PP was seen in TAM (Figure 2), whereas PI showed no difference.

Furthermore, API was also decreased in comparison with control PEM. Next, we compared phagocytic activity of TAM and PBMo derived from the same patients. Corresponding to PP, PI and API, TAM were inferior to those of PBMo. According to this observation, we shad to investigate whether a tumor-derived product

was responsible for the inhibited phagocytosis of TAM. It was found that all tested concentration of crude supernatants cause a marked inhibition of PEM phagocytosis (Figure 3). It should be noted that this inhibition was prominent in presence of lower dose of supernatants. Similarly, pretreatment of PBMo from healthy donors with examined supernatants caused a concentration dependent inhibition of phagocytosis (Figure 3).

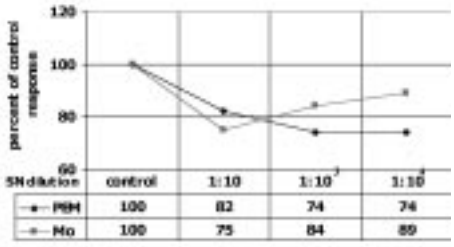


**Figure 3.** Effects of crude supernatants on phagocytic activity of mononuclear phagocytes. Mononuclear phagocytes (PBMo and PEM) from control group were pretreated with different concentrations of crude supernatants from short-term cultures of the ascitic cells from patients with PC. Percent of phagocytosis was evaluated using a light microscope. Obtained results were presented as the percent of control response. data are expressed as the mean of ten experiments

In addition, maximal inhibitory activity is observed when PBMo are treated with higher concentration of supernatants, whereas the inhibition declines in presence of lower dose.

*Nitrite determination.* In further experiments we measured plasma and ascitic fluid nitrite levels in patients with peritoneal carcinomatosis. Our data demonstrate an elevation in levels of PL and AF nitrite among patients with peritoneal carcinomatosis (Figure 4).

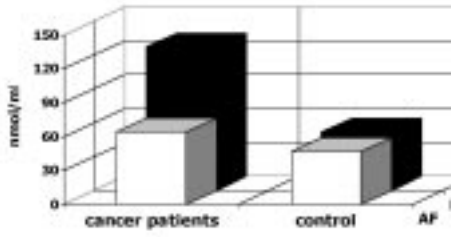


**Figure 4.** Plasma and ascitic fluid nitrite levels. Nitrite concentrations in plasma (PL) and ascitic fluid (AF) were determined by Griees reaction. Elevated plasma and ascitic fluid nitrite level were observed among cancer patients with peritoneal carcinomatosis. Values represent the mean of duplicate samples from ten different experiments.

In addition, we examined NO production in PBMo and TAM derived from patients with peritoneal carcinomatosis. As shown in Figure 5. PBMo from cancer patients produce higher levels of NO than PBMo from healthy control, although statistical significant was P>0.05. More importantly, TAM display a diminished capacity to produce NO and fourfold decrease in NO production was observed (Figure 5). This result may suggest that tumor growth promotes distal Mo suppressor activity by increasing Mo nitric

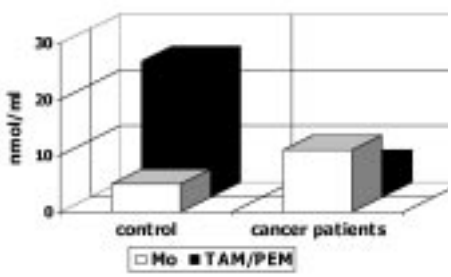


**Figure 5.** NO secretion by mononuclear phagocytes. Mononuclear phagocytes ($0.2x10^6$ cells/well) from controls (Mo and PEM) and cancer patients (Mo and TAM) were cultured for 24h in plain medium. NO production was evaluated in cell supernatants as nitrite by Griees reaction. Values represent the mean of duplicate samples from ten different experiments

oxide production and concomitantly down-regulating the local production of these cytotoxic molecules.

## DISCUSSION

Although malignant tumors may be immunogenic, and immunosurveillance may limit the outgrowth of some tumors, it is unfortunately clear that the immune system does not prevent frequent occurrence of lethal human cancer (31). There is strong evidence that during neoplasm's development, tumor cells either directly or indirectly (by soluble immunomodulating molecules) may induce systemic disorders of many important immunological functions (32,33,40). Monocyte / macrophages have a wide array of functions critical to host defense, and tumor growth may alter these complex mechanisms resulting in the functional inability of the macrophages. Tumor-associated macrophages have been extensively studied, yet the pleiotropic, ambivalent function of these cells still remains a mystery. This functional heterogeneity occasionally results in mutually antagonistic functions. For example, TAM can promote the growth of tumor cells *in vitro*. On the other hand, TAM can be activated to destroy neoplastic cells and inhibit tumor growth (34).

The investigation was designated to evaluate numeric and functional properties of mononuclear phagocytes from cancer patients with peritoneal carcinomatosis. We found that cancer patients showed marked peripheral blood neutrophilia and lymphocytopenia. Several laboratories have demonstrated a prominent neutrophilia and lymphocytopenia in the presence of developed tumors (35-37). Moreover, Nicolini et al. recently showed that the decrease of circulating $CD8^+$ cells in cancer patients is compatible with the hypothesis of $CD8^+$ cells localization at the site of micrometastases (38). We previously showed that $CD8^+$ cells represent a major component of the breast cancer lymphoreticular infiltrates (39). These data correlate with those of Nicolini and may explain profound lymphocytopenia observed in our experimental model.

Next, we have shown that contrary to PBMo which preserve their phagocyte function, TAM exhibit significant inhibition of these complex mechanisms. According to this observation, we wanted to investigate whether a tumor-derived product was responsible for the inhibited phagocytosis of TAM. Our results suggest that a tumor-derived soluble factors are responsible for quantitative differences noted in TAM and PBMo from cancer patients. Alternatively, TAM disjunction may be secondary to the maturational stages of these cells. Considerable progress has been made in the definition of the origin, regulation and function of TAM, although phagocytic function of TAM has been less extensively studied and remains obscure. Yet, Adachi et al. 1993 showed that the highest degrees of TAM phagocytosis are associated with favorable prognosis (17). Moreover, Fiumara et al. (1997) demonstrated that distant metastases developed in none of the cancer patients with *in situ* evidence of neoplastic cells phagocytosis by TAM (18). We found that TAM from metastatic lesion display profound impairment of the phagocytic function. These data, together with those of Fiumara et al. indicate that non-specific immune reactions involving neoplastic cells' phagocytosis by macrophages, play an important role in the development of distant metastases in cancer patients.

In further studies we evaluate the pattern of NO production in ten advanced cancer patients. We therefore measured plasma and ascitic fluid nitrite levels as an index of *in vivo* NO production. We observed almost threefold increase in NO plasma levels in patients with peritoneal carcinomatosis. Thus, we conclude that the higher levels of nitrites reflect endogenous generation of NO resulting from progression of peritoneal carcinomatosis. To ascertain the role of mononuclear phagocytes in these alterations, we examined NO production in PBMo and TAM obtained from patients with peritoneal carcinomatosis. We have found that PBMo from cancer patients produce NO at levels comparable or higher to those of PBMo from healthy individuals. On the other hand, TAM display a depressed capacity to produce NO, an important citolytic factor. However, there is a large body of evidence that during tumor progression, complex process leading to NO production may become altered, resulting in the inability of the macrophages to kill tumor cells (40-43). Our results concur with those of Sotomayor et al. (41)

and Di Napoli et al. (42) who have demonstrated a suppression of NO production in TAM. However, the molecular event leading to the reduced production of NO by TAM have not been elucidated, although Di Napoli et al. have demonstrated reduced expression of the inducible nitric oxide synthase (iNOS) gene in tumor bearer macrophages (44). Subsequently, this reduced rate of iNOS gene transcription was shown to be due, at least in part, to the action of tumor-derived phosphatidyl serine (43,44). However, the present study suggests that the development of distant metastases can suppress host macrophage antitumor activity through alteration in phagocytic activity and NO production.

There are several possible explanation for the functional impairment of TAM. First, there is strong evidence that during neoplasm's development factor produced by the tumor (transforming growth factor-β, prostaglandin-$E_2$, interleukin-10, and phosphatidyl serine) may lead to dysregulaton of complex macrophage tumoricidal machinery (32,45,46) . Second, neoplastic cells have the capacity to impair the production of T cells derived cytokines (interferon-γ) important in macrophage activation. However, it is possible that tumor progression may result in changes in T cells subpopulations, such as decrease in the number or function of $Th_1$ cells, with concomitant increase of $Th_2$ cells. In fact, the shift from a predominant $Th_1$ to a $Th_2$ phenotype during the tumor progression has been questioned in one (42) and supported in other studies (47). Lastly, macrophages are functionally heterogeneous, owing in part to differences in stage of differentiation and activation, and in part to local microenvironmental influences. Hence, cells or factors within the tumor may have induced the infiltrating Mo to have remained in, or to have differentiated into a state less capable of responding to activated agents (48).

Generally, we have demonstrated that qualitative and quantitative differences exist in the alterations noted in TAM and PBMo from cancer patients. Moreover, these results suggest that tumor growth promotes downstream Mo suppressor activity by increasing Mo production of NO (Mo derived NO mediated suppression of T cells proliferation, Alleva et al. 1994 (46)) and concomitantly down-regulating the local production of these antitumor molecules by Mph.

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