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PROLIFERATION OF NAIVE T LYMPHOCYTES AND T LYMPHOBLASTS IN THE PRESENCE OF TISSUE SPECIFIC MACROPHAGES

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In this study the antigen-presenting ability of tissue specific macrophages isolated from bone marrow, spleen, peritoneal cavity and lungs was analyzed. Murine macrophages were isolated by a one-step adherence procedure (for 24 hours) and pretreated with mytomycin C. The antigen-presenting ability of the macrophages was tested in T cell proliferation assays. The ability of macrophages to support antigen-specific proliferation of T lymphoblasts was investigated when sheep red blood cell (SRBC)-specific T blasts were stimulated in vitro by antigen in the presence of different numbers of tissue specific macrophages. On the other hand, the abilities of macrophages to induce proliferation of naïve T cells were analyzed in allogeneic and syngeneic mixed leukocyte reactions (MLRs). It was demonstrated that tissue specific macrophages supported antigen specific proliferation of T lymphoblasts in vitro. They also induced the activation of allogeneic and syngeneic T cells. Increasing the number of macrophages co-cultured with T cells, led to a certain inhibitory effect on T cell proliferation.

Key words: macrophage, antigen-presenting cells, sheep red blood cells (SRBC), mixed leukocyte reaction (MLR), mouse

INTRODUCTION

Classically, the immune response has been divided into an initial innate and a later adaptive response [Jr Janeway, 1992] and macrophages are included in both parts. As very efficient scavenger cells macrophages phagocytize foreign particles and microorganisms [Davis *et al.*, 1998], while as antigen-presenting cells they are included in the initial phase of the specific immune response [Davis *et al*., 1998]. It is well known that dendritic cells are specialized antigen-presenting cells with the greatest potential to stimulate T-lymphocytes [Massard *et al*., 1996]. Data related to the antigen-presenting ability of macrophages are not consistent as, it was shown that macrophages could either activate [Lee and Wong, 1982; Davis *et al*., 1998; Lipscomb *et al*., 1981], or suppress [Holt *et al*., 1988; Holt, 1986] T cell proliferation.

Although macrophages originate from a common bone marrow progenitor population [Van Furth and Sluiter, 1983], resident tissue macrophages display considerable heterogeneity [Forster and Landly, 1981; Laskin *et al*. 2001]. In this study we analyzed the ability of murine tissue specific macrophages to induce T cell proliferation *in vitro*. The antigen-presenting ability of macrophages from bone marrow, spleen, peritoneal cavity and lungs was tested. Their ability to activate sheep red blood cell (SRBC)-specific T lymphoblasts and/or allogeneic and syngeneic naïve T cells in a mixed leucocyte reaction (MLR) was investigated.

T cell proliferation was found in the presence of macrophages from different sources, with the highest T cell proliferative rate obtained in antigen specific MLRs. An inhibitory effect on T cell proliferation was achieved by increasing the number of macrophages.

MATERIALS AND METHODS

Animals. 6-8 week old male or female mice (C57BL/6 and CBA) were obtained from the Medical Military Academy, Belgrade.

Macrophage enrichment procedure. Cell suspensions from bone marrow, spleen, peritoneal cavity and lungs were prepared as previously described [Zivancevic-Simonovic *et al*., 2004]. Following isolation, the cells were centrifuged at 200 x g for 10 min, and suspended in RPMI 1640 medium (Gibco), containing 100 IU/ml of penicillin, 100 μg/ml of streptomycin, 2 mM L-glutamine, and 10% fetal calf serum (FCS, Gibco). Macrophage enrichment was achieved by a simple-one-step adherence procedure. Cell suspensions were plated on to 100 mm plastic Petri dishes and incubated at 37^o C in 5% CO_2 (incubator, Hereaus) for 24 hours. Adherent cells were removed from the plastic surface by incubating with PBS containing 0.02% disodium EDTA for 20 min at 4^{o}C. Cells were washed twice with PBS, resuspended in RPMI 1640 medium and incubated (37^{o}C, 5% CO_2) with mitomycin C (25 μg/ml, Sigma). After 20 min, the cells were washed three times in RPMI 1640 medium and resuspended at the final concentration of 1×10^5 cells/ml.

Antigen and immunization. C57BL/6 mice were immunized with SRBC in the hind foot pads and base of the tail. The first, second, and third injections were applied weekly in complete and incomplete Freund's adjuvant and phosphate-buffered saline (PBS), respectively.

T cell isolation. T lymphoblasts were isolated from the periaortic, popliteal, and inguinal lymph nodes of immunized mice on a nylon wool column [Kappler and Marrak, 1977] 7 days after the last injection. An overnight incubation at 37^{o}C in a 5% CO_2 atmosphere, in RPMI 1640 medium with 10% FCS, was performed to remove the adherent cells. The nonadherent cells were used for the proliferation assay.

Naïve T cells were isolated by nylon wool filtration [Kappler and Marrak, 1977] of lymph node cells from nonimmunized CBA (for allogeneic MLRs) or C57BL/6 (for syngeneic MLRs) mice.

In vitro cell proliferation assay. T lymphoblasts (4×10^5/well) were stimulated for 96 hrs with 1×10^6 SRBC. The cell proliferation assay was performed in the

presence or absence of mitomycin C-pretreated macrophages (1 x 10^3, 5 x 10^3 or 1 x 10^4 cells/well) in 96-well round-bottom microtiter plates. Allogeneic MLRs were established by adding C57BL/6 macrophages (1 x 10^3, 5 x 10^3 or 1 x 10^4 cells/well) to 4 x 10^5 CBA lymphocyte ($CD3^+$) and syngeneic MLRs by adding C57BL/6 macrophages to T cells isolated from lymph nodes of nonimmunized C57BL/6 mice. The assay was performed in 96-well round-bottom microtiter plates in the presence of indomethacin as stated above. Proliferation was measured by adding 1 μCi (0.037 MBq) [^{3}H] thymidine for the last 24 hours of the culture period. Responses were reported as the mean of triplicate tests, counts per minute (cpm) ± SD.

Flowcytometry. For immunofluorescence analysis of CD3, CD4 or CD8 expression on cells isolated by nylon wool filtration, 1x10^6 cells were incubated with rat anti-mouse CD3, CD4 or CD8 MAbs (1 μg/million lymphocytes), for 20 min at 4°C. Cells were washed three times with PBS, containing 2% FCS, and 0.01% NaN_3. Following that, the cells were incubated with FITC-labeled goat-anti-rat antibodies (Sigma) (1 μg/assay) for 30 min. Cells were washed three times with PBS, 2% FCS, 0.01% NaN_3, resuspended in 1% formaldehyde and analyzed on a Flowcytometer equipped with a 488 nm Argon laser light source and a 515 nm band pass filter for FITC-fluorescence. A total of 10 000 events were acquired for analysis using CellQuest software. A histogram plot of FITC-fluorescence (x-axis) versus counts (y-axis) was obtained in logarithmic fluorescence intensity.

RESULTS

It was shown that specific T lymphocytes do proliferate in the presence of antigen and macrophages as antigen-presenting cells. After isolation of lymph node cells, flowcytometry analysis revealed that 96.9% of the nylon wool isolated lymphocytes were CD3-positive (Fig 1). The proportion of CD4+ cells was 65.3% whereas the proportion of CD8+ was 30.7%. When T cells isolated from the lymph nodes of immunized mice were stimulated with three doses of SRBC (1x10^5, 5x10^5 or 1x10^6, data not shown) and different concentrations of macrophages, the highest proliferative response was obtained with 1x10^6 SRBC and all following experiments were done in the presence of that antigen dose.

We demonstrated that macrophages (24-hour adherent) isolated from bone marrow, spleen, peritoneal cavity and alveolus were able to present SRBC to specific T lymphoblasts *in vitro*. Although T cell proliferation was obtained in the presence of all macrophage concentrations used (Fig. 2), the proliferative response was partly diminished with 1x10^4 macrophages/well for all macrophage sources. This inhibitory effect was especially evident with alveolar macrophages.

For allogeneic MLRs, T lymphocytes isolated from lymph nodes of non-immunized CBA mice were cultured with C57BL/6 macrophages (1x10^3, 5x10^3 or 1x10^4). A proliferative response by T lymphocytes was obtained (Fig. 3), although increasing the number of macrophages had an inhibitory effect on T cell proliferation. In comparison with other macrophage sources, alveolar macrophages at 1x10^4 macrophage/well exerted the strongest inhibitory effect.

For syngeneic MLRs, macrophages from bone marrow, spleen, peritoneal cavity and alveolus all induced T cell proliferation (Fig. 4). Increasing the number of macrophages in the culture from $1x10^3$ to $5x10^3$/well induced proliferation enhancement in three out of the four cases. Lower T cell proliferative responses were obtained with macrophage concentration of $1x10^4$/well.

Thus, an inhibitory effect on T cell proliferation by increasing the number of macrophages was observed both for T lymphoblasts stimulated by antigen and for naïve T cells co-cultured with allogeneic or syngeneic macrophages.

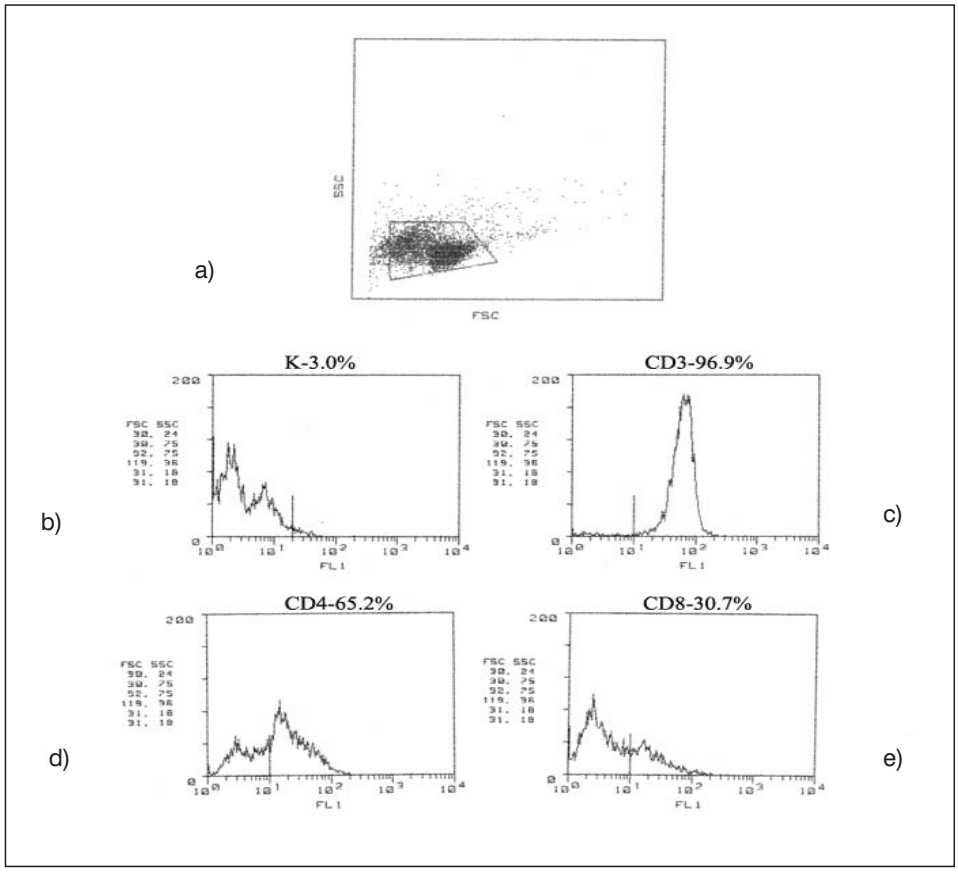

Figure 1. Immunofluorescence analysis of lymph node cells isolated on nylon wool. Forward and side scattering characteristics of the cells (a), nonspecific binding (b), CD3 expression (c), CD4 expression (d), and CD8 expression (e). Histogram plots of FITC-fluorescence (x-axis) versus counts (y-axis) in logarithmic fluorescence intensity are shown

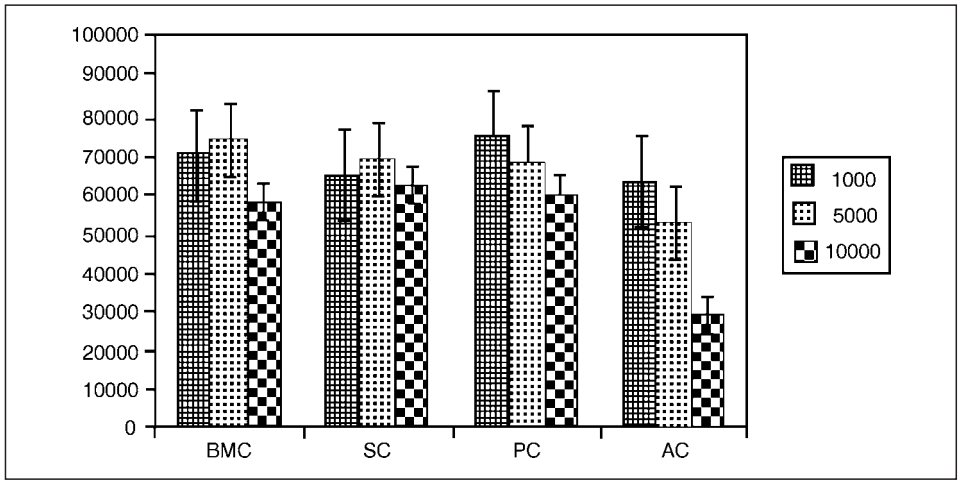

Figure 2. Antigen specific proliferation of T lymphoblasts in the presence of $1x10^6$ SRBC and $1x10^3$, $5 x10^3$, or $1 x10^4$ tissue specific macrophages as antigen-presenting cells. Macrophages were isolated as 24-hour adherent bone marrow cells (BMC), spleen cells (SC), peritoneal cells (PC) and alveolar cells (AC). Column height is the mean of triplicate cultures, counts per minute (cpm) ± SD

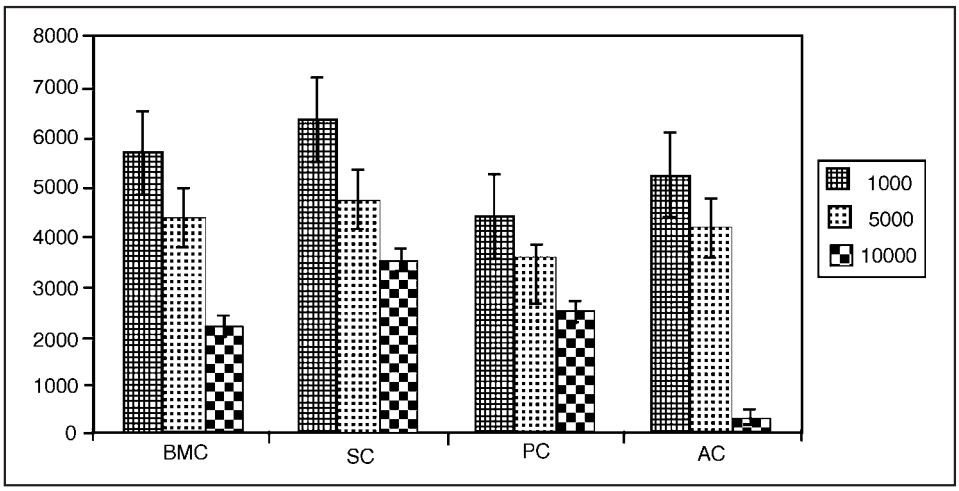

Figure 3. T cell proliferation in allogeneic MLRs. Proliferative response of lymph node T cells, isolated from CBA mice, in the presence of $1x10^3$, $5 x10^3$, or $1 x10^4$ C57BL/6 macrophages obtained as 24-hour adherent bone marrow cells (BMC), spleen cells (SC), peritoneal cells (PC) and alveolar cells (AC). Column height is the mean of triplicate cultures, counts per minute (cpm) ± SD

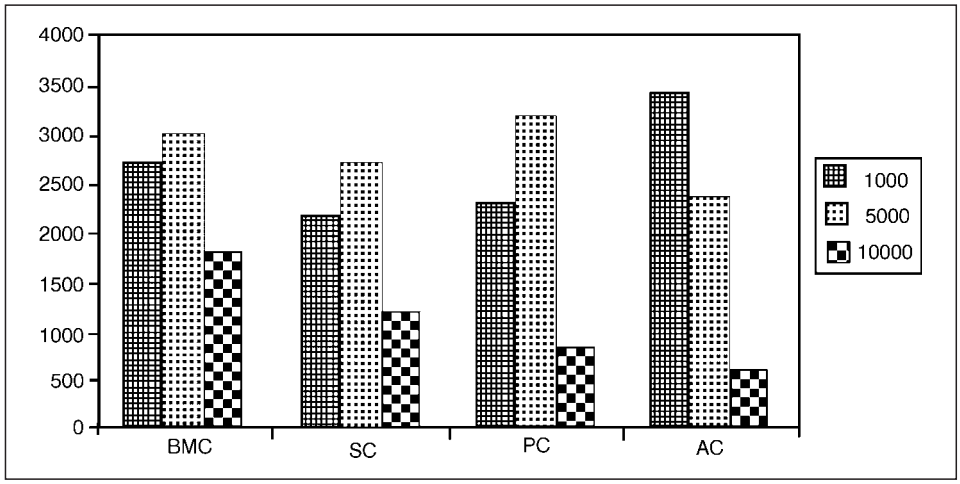

Figure 4. Fig. 4. T cell proliferation in syngeneic MLRs. Proliferative response of lymph node T cells, isolated from C57BL/6, mice in the presence of $1x10^3$, $5 x10^3$, or $1x10^4$ C57BL/6 macrophages obtained as 24-hour adherent bone marrow cells (BMC), spleen cells (SC), peritoneal cells (PC) and alveolar cells (AC). Column height is the mean of triplicate cultures, counts per minute (cpm) ± SD.

DISCUSSION

In this study we analyzed the ability of tissue specific macrophages to support activation of T lymphoblasts or naïve T cells. It was shown that macrophages isolated from bone marrow, spleen, peritoneal cavity and alveolus supported antigen-specific activation and induced proliferation of allogeneic or syngeneic lymphocytes. The macrophages were isolated by a one-step adherence procedure lasting 24 hours in order to remove dendritic cells which adhere transiently and become non-adherent after 16 hours in culture [Crow and Kunkel, 1982; Knight *et al*., 1986]. It was shown that more than 95% of 24-hour adherent cells were macrophages [Zivancevic-Simonovic *et al*., 2004]. Accordingly, the antigen-presenting ability in our experiment was not mediated by dendritic cells well known as effective APCs [Massard *et al*., 1996] and necessary for naïve T cell activation [Hart, 1997; Banchereau and Steinman, 1998; Steinman and Witmer, 1978].

Dendritic cells (DC), macrophages and B cells are "professional" APC but each of these APC possess unique characteristics that favour or restrict presentation of antigen to T cells [Syme *et al*., 2002]. Having this in mind, we chose SRBCs as antigens in our experimental model and investigated if macrophages had the ability to present that large size, particulate antigen to specific T cells. Although it was shown [Malynn and Wortis, 1984] that antigen-specific B cells played a role in the induction of T cell proliferative responses to

SRBC antigens, especially that antigen-specific B cells were necessary at low doses of SRBC [Malynn *et al*., 1985], we showed that macrophages isolated from bone marrow, spleen, peritoneal cavity and alveolus could support antigen-specific proliferation of T cells in the presence of high doses ($1x10^6$) of SRBC. As T cells were isolated from lymph nodes of previously immunized mice, we concluded that macrophages are efficient in presenting antigen to T lymphoblasts.

The literature data regarding antigen-presenting ability of macrophages are rather different and dependent on the source of macrophages and the nature of the antigen. It was shown that murine macrophages isolated from the spleen [Geijtenbeek *et al*., 2002], as well as bone marrow-derived macrophages [Lee and Wong, 1982] possessed antigen-presenting ability. There are some contradictory data about the functioning of alveolar macrophages as antigen-presenting cells. Depending on animal source as well as the antigen used, alveolar macrophages either support [Lipscomb *et al*., 1981] or quit [Franke-Ullmann *et al*., 1996] antigen-specific T cell proliferation. In contrast to previous reports of either stimulatory or inhibitory effects of lung macrophages on lymphocyte function, Liu *et al*. [1984] demonstrated that the proliferative response was a complex function of T lymphocyte and macrophage concentrations. They also showed that in the presence of a low concentration of lymphocytes, a low macrophage concentration enhanced proliferation, whereas a higher concentration inhibited proliferation. In the presence of high concentrations of lymphocytes, macrophages only inhibited T cell proliferation. In our experiment, an inhibitory effect on T cell proliferation was obtained. This effect was shown in the presence of all macrophage populations, but was particularly obvious with alveolar macrophages as the antigen-presenting cells. This might be partly explained by the fact that macrophages are a heterogeneous population, even within a particular tissue [Laskin *et al*., 2001]. It was demonstrated [Lee and Wong, 1982] that only small macrophages induced an enhancement in antigen presentation, whereas large activated macrophages exerted an immunosuppressive effect that probably neutralized any augmentation of stimulatory activity. This inhibitory effect might be a consequence of macrophage heterogeneity, as we have already showed differences in size of 24-hour isolated tissue specific macrophages [Zivancevic-Simonovic *et al*., 2004].

The mixed lymphocyte reaction (MLR) has been used to elucidate naïve T lymphocyte proliferation [Hart, 1997; Banchereau and Steinman, 1998; Steinman and Witmer, 1978; Massard *et al*. 1996]. Although, it is generally accepted that DC stimulate an allogeneic [Steinman and Witmer, 1978; Crow and Kunkel, 1982] or syngeneic [Crow and Kunkel, 1982; Guidos *et al*., 1984] MLR proliferation, the role of macrophages in MLRs is not completely clarified. Therem are data indicating stimulatory [Minami *et al*., 1980], enhancing [Naito *et al*., 1989] and inhibitory effects [Smolen *et al*., 1981] of macrophages on MLRs. In our experimental model macrophages were able to induce allogeneic as well as syngeneic T cell proliferation and an inhibitory effect on T cell proliferation by increasing the number of macrophages was obtained in MLRs. The results presented in this work support the thesis that, depending on the type and number, activation state,

interaction and communication with T cells, macrophages could provide either immunostimulatory or immunosuppressive effects on T cells.

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PROLIFERACIJA NAIVNIH I AKTIVIRANIH T LIMFOCITA U PRISUSTVU TKIVNO SPECIFIČNIH MAKROFAGA

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SADRŽAJ

U ovom radu je ispitivana sposobnost mišjih makrofaga izolovanih iz kostne srži, slezine, peritonealne šupljine i alveola da prezentuju antigen i indukuju proliferaciju T limfocita. Makrofazi su izolovani adherencijom tokom 24-časovne kulture i pretretirani mitomicinom C. Da bi se ispitala sposobnost makrofaga da indukuju proliferaciju limfoblasta, T limfociti specifični za ovčje eritrocite izolovani iz limfnih čvorova imunizovanih miševa u *in vitro* uslovima, su restimulisani antigenom u prisustvu tkivnih makrofaga izolovanih iz različitih tkiva. Pored toga, sposobnost makrofaga da indukuju proliferaciju *in vivo* naivnih T limfocita ispitivana je u mešanoj kulturi tkivnih makrofaga i alogenih ili singenih limfocita. Dokazano je da tkivni makrofazi izolovani iz kostne srži, slezine, peritonealne šupljine i alveola mogu da potpomognu antigen-specifičnu proliferaciju T limfoblasta u *in vitro* uslovima, kao i da aktiviraju naivne alogene i singene T limfocite. Povećanje broja makrofaga dovelo je do smanjenja T limfocitne proliferacije.