Endocytic and NBT-reduction activities and TNF expression by macrophages and monocytes of the armadillo (*Dasypus novemcinctus*)

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Summary

The armadillo could be considered as an experimental model for leprosy studies. *Mycobacterium leprae* main host cells are macrophages and Schwann cells. However, endocytosis and germicidal activities of armadillo macrophages and monocytes have not yet been evaluated. The aim of this research was to evaluate endocytosis and NBT-reduction activities of monocyte-derived macrophages (MDM) and monocytes from non-infected armadillos, as well as to determine tumor necrosis factor (TNF) levels during stimulation with phorbol myristate acetate (PMA). Mononuclear cells of peripheral blood were purified using the ficoll-hypaque technique. The monocytes obtained were transformed to MDM after being incubated for 8 days in an RPMI-1640 medium supplement with fetal calf serum at 37°C. Endocytosis and NBT-reduction were evaluated by yeast ingestion. TNF production by stimulation with PMA was evaluated through a bioassay with L-929 cells. The results revealed an endocytosis of 71.77 ± 4.11% and 60.66 ± 6.9% ($P<0.001$) for armadillo MDM and monocytes respectively, whereas NBT-reduction was 16.57 ± 4.79% and 17.66 ± 4.08%, respectively. With the addition of recombinant human IFNγ for 24 h, endocytosis and NBT reduction by MDM were 89.92 ± 6.17% ($P<0.001$) and 36.42 ± 4.31% ($P<0.001$), respectively. Low levels of TNF are produced (10 U/ml) when MDM and armadillo monocytes are stimulated with 25 and 50 ng/ml of PMA, in comparison with human U-937 cells. In conclusion, MDM and armadillo monocytes show diminished activities in oxidative bactericidal mechanisms and in TNF production when stimulated with PMA.

Key words: *Dasypus novemcinctus* macrophages, Immune response of armadillo, Monocyte-derived macrophages, NBT reduction, Superoxide anion

Introduction

The armadillo, being the only animal species naturally infected with *Mycobacterium leprae*, is considered as an experimental model for the study of leprosy (Kirchheimer and Storrs, 1971; Truman *et al*., 2011). The main host cells of *M. leprae* are macrophages and Schwann cells (Scollard *et al*., 2006). Macrophages are cells that engulf and kill a wide variety of microbes efficiently. Furthermore, they are involved in a number of key physiological processes and complex responses such as processing antigen and presenting it to T cells, as well as the production of proinflammatory cytokines (Labro, 2000; Sharma *et al*., 2013). Blood monocytes are considered as circulating precursors of macrophages and dendritic cells, and together with the latter, they have collectively been termed as mononuclear phagocyte system (Chang, 2009). When monocytes are cultured *in vitro* for several days, these cells increase progressively in size and complexity, and transform into macrophages (Sutton and Weiss, 1966; Pinet *et al*., 2003). Human MDM have been shown to retain their ability to ingest several particle types, such as latex particles, sheep erythrocytes, and yeast (Sutton and Weiss, 1966; Sasada *et al*., 1987). However, unlike monocytes, MDM exhibit less microbicidal activity (Sutton and Weiss, 1966; Sasada *et al*., 1987; Chang, 2009). To date, endocytic and germicidal activities of armadillo macrophages have not been evaluated. The aim of this investigation was to evaluate endocytic and microbicidal activities of armadillo monocyte-derived macrophages (armadillo MDM) as well as to determine tumor necrosis factor levels (TNF) when these cells are stimulated with phorbol myristate acetate (PMA).

Materials and Methods

Animals

Nine-banded armadillos (*Dasypus novemcinctus*) were captured in the state of Michoacán in Mexico. Twenty milliliters of peripheral blood were collected from the anesthetized animals by cardiac puncture, as described previously by López-Hurtado *et al*. (2005).

Cell lines

U-937 human myelomonocytic (ATCC, CRL-1593.2) and L929 mouse subcutaneous connective tissue cells

(ATCC, CCL-1) were used. All cell lines used in the present study were maintained in RPMI-1640 medium (Sigma, St. Louis, MO, USA) containing 10% FBS and antibiotics (Invitrogen, Grand Island, NY, USA).

Monocytes preparations

Total leukocytes were isolated by dextran sedimentation and mononuclear cells were purified by the ficoll-hypaque (Sigma) technique (López-Hurtado *et al*., 2005). Obtained mononuclear cells were washed twice in an RPMI-1640 medium and resuspended to a concentration of 1×10^6 cells/ml with an RPMI-1640 medium supplemented with 10% fetal calf serum and antibiotics (RPMI-complete); 1 ml of the mononuclear cells was added to each well of a 24-well microplate and incubated for 90 min at 37°C in a humidified 95% air/5% CO_2 atmosphere. The microplates were vigorously washed five times with RPMI-complete to remove non-adherent cells. Adherent cells were then exposed to ice-cold Hank's balanced salt solution (SSBH, Sigma) supplemented with 0.2% bovine serum albumin and 0.1% EDTA (Sigma) for 30-45 s, and then gently removed from the surface with a rubber policeman. These cells were then washed three times with SSBH. Viability was greater than 95% according to the trypan blue exclusion test. The purity of these preparations was greater than 90% as assessed by Wright stain morphology.

Monocyte-derived macrophages (MDM)

An armadillo monocyte suspension was added to a 24-well microplate (5×10^4/ml per well) provided with glass coverslips. All microplates were incubated at 37°C with 5% CO_2 for 8 days to allow the differentiation of monocytes from macrophages. The RPMI-complete was changed every 4 days.

Endocytosis assay and NBT dye reduction assay

Endocytosis and the reduction of nitroblue tetrazolium dye (2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-[3,3'-dimethoxy-4,4'-diphenylene]-ditetrazolium chloride (Sigma) evaluation was carried out using the macrophages adhering to the glass coverslips. These assays were performed as previously described by Rojas *et al*. (1996) and López-Hurtado *et al*. (2009). One milliliter of an opsonized yeast suspension (*Saccharomyces cerevisiae*, at 30×10^6 cells per milliliter in RPMI culture medium) and 0.5 ml of a 0.2% nitroblue tetrazolium dye solution (NBT) in 0.85% NaCl were added to each well of the microplate and incubated for 90 min. The coverslips were then removed, rinsed 3 times in physiological saline solution, stained for 10 min with 0.5% safranin in water, rinsed in distilled water, air dried, and mounted on glass slides using synthetic resin. The degree of endocytosis and NBT dye reduction was evaluated by observation under a microscope by counting 200 cells in duplicate preparations. The effect of interferon-gamma (IFN-γ) on endocytic and NBT-reduction activities was also evaluated after 24 h of incubation, with 1 ng/ml of recombinant human IFN-γ (Sigma) as described previously.

TNF activity detection

TNF detection was performed through a bioassay using L929 cells, as previously described by Guerra-Infante *et al*. (2001). One milliliter of RPMI-complete supplemented with PMA (Sigma) at 25, 50, and 100 ng/ml was added to each well of a 24-well microplate containing macrophages in coverslips. After 24 h of incubation at 37°C in 5% CO_2, the culture supernatants were recovered and maintained at -70°C until used. TNF detection was carried out by adding 50 µl of the culture supernatants to each well in triplicate of a 96-well flat-bottom microplate that already contained 20×10^3 L-929 cells/well. Then, 50 µl of RPMI-complete supplemented with 1 µg/ml of actinomycin D (Sigma) was added to each well. Culture supernatants of U-937 cells, stimulated under the same conditions as those of the armadillo macrophages, were used as the control. The microplate was incubated at 37°C for 24 h in 5% CO_2. Supernatants were aspirated and 100 µl of methanol was added. After 7 min of incubation, 50 µl of 2% crystal violet was added. After 10 min, the microplate was washed five times with a PBS solution at 200 µl per well. Then, 100 µl of 33% glacial acetic acid was added. Finally, the microplate was read in a microplate reader at 600 nm for absorbance (ELx800, Bio-Tek Instruments, Inc., Winooski, VT, USA). The cytotoxic activity (in units per milliliter) was defined as the reciprocal of the dilution resulting in 50% cytotoxicity, determined by plotting the regression line of the dilution against absorbance.

Statistical analysis

The results were analyzed by a non-parametric Mann-Whitney U statistical test, with a $P<0.05$ considered to be statistically significant.

Results

The results of this study showed that in terms of morphology, the purity of the obtained armadillo monocytes was 90%. These cells were incubated for 8 days at 37°C in a 5% CO_2 atmosphere. After the incubation period, cells showed morphological changes, such as size increases of 5 µm to approximately 12 µm, as well as centric and eccentric nuclei (Figs. 1A, B). The endocytic capacity of armadillo MDM was 71.77 ± 4.11%, whereas in recently purified monocytes, it was 60.66 ± 6.9% (Table 1), with a statistically significant difference between the groups ($P<0.01$). The ability of armadillo MDM to achieve NBT reduction was 16.57 ± 4.79%, whereas in monocytes it was 17.66 ± 4.08%. but these differences were not statistically significant (Table 1).

The addition of recombinant human IFN-γ (rhIFN-γ) to armadillo MDM for 24 h turned out to be biologically active on these cells because it induced an increase in endocytosis (89.92 ± 6.17%) and in NBT reduction (36.42 ± 4.31%). These increases were statistically

significant (P<0.001) as compared to the armadillo MDM which was not stimulated with rhIFN-γ (Table 1).

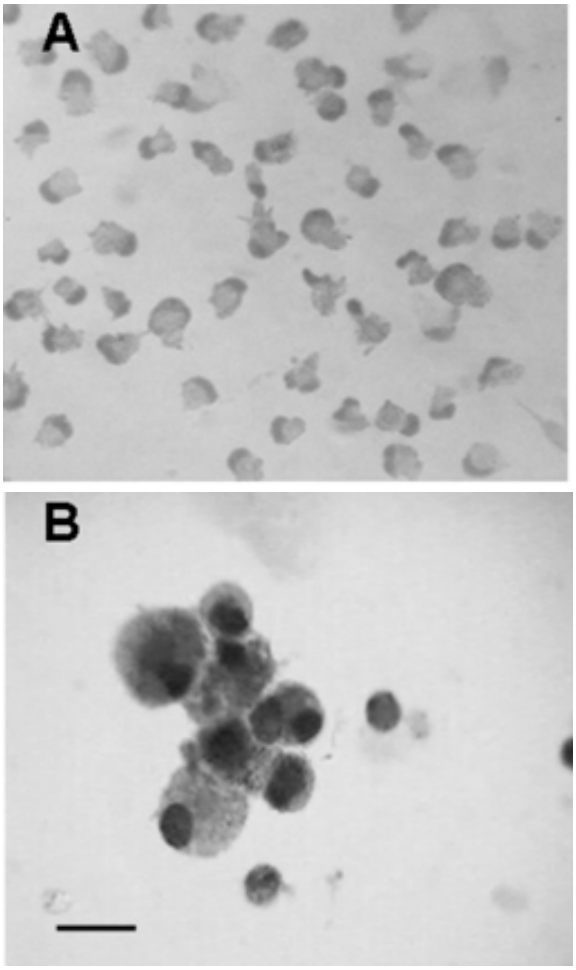

Fig. 1: Morphology of armadillo monocyte-derived macrophages. (A) Armadillo monocytes incubated at 37°C in 5% of CO_2 by 24 h. (B) Armadillo monocytes incubated at 37°C in 5% of CO_2 by 8 days, these cells were considered as monocyte-derived macrophages (MDM). The line represents 24 µm

Table 1: Percentage of endocytosis and NBT reduction of MDM

Cell types	Percentage of endocytosis	Percentage of NBTr[c]
Monocytes (n=5)	60.66 ± 6.9[b]	17.66 ± 4.08
MDM (n=50)	71.77 ± 4.11[A]	16.57 ± 4.79
MDM-stimulated[a] (n=5)	89.92 ± 6.17[A]	36.42 ± 4.31[A]

[a] Monocyte-derivied armadillo macrophages stimulated with recombinant human interferon-γ by 24 h. [b] Median and standard deviation. [c] Percentage of nitroblue-tetrazolium reduction. [A] Significant difference compared to armadillo monocytes by U-Mann-Whitney test P<0.001

The detection of TNF in culture medium supernatants of armadillo MDM stimulated with PMA is shown in Table 2. TNF-alpha levels of less than 1.0 U/ml were observed in culture supernatants of MDM or monocytic cells when stimulated with 25 ng/ml of PMA, whereas in U937 cells, these levels were 9.8 ± 3.89 U/ml. However, increased TNF-α production in response to 50 and 100 ng/ml of PMA was observed in armadillo monocytes that showed TNF activity of 2.25 ± 0.45 and 8.06 ± 1.39 U/ml, while armadillo MDM showed TNF activities of 3.89 ± 1.44 and 11.9 ± 2.38 U/ml, and the U937 cells line showed TNF activities of 11.4 ± 3.30 and 14.4 ± 4.2 U/ml, respectively.

Discussion

In this study, after incubating armadillo monocytes for a period of 8 days, these cells showed morphological changes, such as increased size and centric and eccentric nuclei, as occurs with macrophages from other animal species (Schlesinger *et al*., 1984; Sasada *et al*., 1987; McCullough *et al*., 1999; Chan, 2002); based on these changes, the cells were considered as macrophages.

In the case of endocytic activity and NBT reduction, armadillo monocytes and MDM showed adequate endocytic activity but low NBT-reduction capacity, as compared to armadillo neutrophiles (Rojas *et al*., 1996; López-Hurtado *et al*., 2005). NBT is a dye that evaluates the NADPH oxidase system, a multiprotein complex that acts as the main source of reactive oxygen species such as superoxide anion, hydrogen peroxide, hydroxyl radicals, and singlet oxygen, which constitute the oxygen-dependent microbicidal arsenal of phagocytes (Nakagawara *et al*., 1981; Ostanin *et al*., 2007).

In particular, the NBT dye is reduced and precipitates when macrophages and monocytes produce the superoxide anion (Nakagawara *et al*., 1981). It is not surprising that armadillo MDM produce low levels of superoxide anions since studies by Schlesinger *et al*. (1984) showed that macrophages and multinucleated giant cells derived from human monocytes had a decreased ability to reduce NBT (Schlesinger *et al*., 1984). This low superoxide anion level produced by armadillo MDM is probably due to incubation for long (3 days or more) periods of time (Nakagawara *et al*., 1981; Schlesinger *et al*., 1984). Similarly, Nakagawara *et al*. in 1981, reported a 60% diminution of superoxide anion and hydroxyl radical concentrations when macrophages are stimulated with PMA or opsonized zymosan for 3 days (Nakagawara *et al*., 1981). A diminished NBT-reduction value of monocytes and MDM from armadillo indicates that these cells might, in part, produce low

Table 2: Levels of TNF in culture supernatants of armadillo cells stimulated with PMA

PMA concentration (ng/ml)	TNF in Monocytes (n=5) (U/ml)	TNF in MDM[a] (n=5) (U/ml)	TNF in U-937 cells (n=10) (U/ml)
25	<1.0[*]	<1.0[*]	9.8 ± 3.89
50	2.46 ± 0.49[b*]	3.89 ± 1.44[*]	11.4 ± 3.30
100	8.06 ± 1.39[*]	11.9 ± 2.38	14.4 ± 4.20

[a] Monocyte-derived macrophages. [b] Median and standard deviation of units of TNF by milliliter. [*] Significant difference compared to U-937 cells by U-Mann-Whitney test P<0.025

levels of superoxide anions.

In the early 1980s, IFN-γ was established as the most common signal used for activating macrophages (Young and Hardy, 1995; Misch *et al*., 2010). In this study, we investigated the effect of rhIFN-γ on endocytic and NBT reduction activities of armadillo MDM which resulted in an increase in endocytosis and rNBT of these cells. This is because IFN-γ is a macrophage activating factor that induces a greater antimicrobial activity in these cells due to nitric oxide synthesis (Knowles and Moncada, 1994; James, 1995). Nitric oxide is a product of the conversion of arginine to citrulline, carried out by nitric oxide (NO) synthase. Nitric oxide synthesis requires this substrate and other coenzymes/cofactors in the presence of calmodulin and NADPH (Knowles and Moncada, 1994). The need to use NADPH for the NO synthesis could explain why there was an increased NBT reduction by armadillo MDM when stimulated with rhIFN-γ. Besides, the obtained results suggest that rhIFN-γ can induce NO synthase gene transcription in armadillo MDM. Notwithstanding that, Peña *et al*. (2008) described armadillo macrophages' inability to produce measurable levels of NO when induced by recombinant armadillo IFN-γ (Peña *et al*., 2008).

The IFN-γ gene has been widely cloned and characterized from mammals and other vertebrates. The IFN-γ gene from the armadillo was cloned and over-expressed in *Escherichia coli* and it is approximately 62% identical in its amino acid sequence to human IFN-γ (Peña *et al*., 2008). This could explain the stimulation of armadillo MDM with recombinant human IFN-γ. In the present work we have shown that armadillo MDM produced low levels of TNF in comparison with U937 cells stimulated with PMA.

Phorbol myristate acetate (PMA), a biologically active compound derived from a plant, can activate protein kinase C, an enzyme that controls the function of other proteins (Azzi *et al*., 1992; Hug and Sarre, 1993). Protein kinase C (PKC) is a family of serine threonine kinases that play important roles in the regulation of normal cell growth and differentiation (Azzi *et al*., 1992; Hug and Sarre, 1993). Furthermore, PMA induces the expression of TNF in armadillo neutrophils (Guerra-Infante *et al*., 2001).

The difference in the level of TNF production between armadillo MDM and U937 cells stimulated with PMA suggests a reduced ability of armadillo monocytes and MDM to produce TNF. The reason could either be the prolonged exposure to PMA, resulting in a drastic decrease in TNF production (refractory phenomenon), or a defective phosphorylation of PKC (alpha, beta, delta) in the cytosol of these macrophages.

We conclude that armadillo MDM have an adequate endocytic activity but a weak oxidative burst and low levels of TNF production. Due to the latter limitations, it the armadillo could become highly susceptible to experimental infection with *M. leprae*. However, Peña *et al*. (2008) showed that armadillo MDM stimulated with recombinant armadillo IFN-gamma were unable to inhibit the *M. leprae* metabolic activity, while markedly limiting the growth of the intracellular protozoan (*Toxoplasma gondii*) over a 20 h period. In a murine model of *T. gondii* infection, it was demonstrated that IFN-gamma induces TNF production and the anti-toxoplasmic effect provided by IFN-gamma seemed to be partly dependent on the production of TNF (Chang *et al*., 1990).

In a mouse model, peritoneal macrophages produce a less robust oxidative burst against live *Mycobacterium lepreamurium* as compared with a similar number of heat-killed bacteria or with *Mycobacterium microti* (Brett *et al*., 1988). This suggests that armadillo macrophages show a very weak oxidative burst against *M. leprae*, reflected as the increased intracellular survival of this bacterium. However, more research is necessary to corroborate and ascertain the causes of these deficiencies in armadillo MDM.

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