Histol Histopathol (2008) 23: 573-581

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

In situ detection and distribution of inflammatory cytokines during the course of infection with *Nocardia brasiliensis*

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Summary. Actinomycetoma, caused by the intracellular bacterium *Nocardia brasiliensis*, is characterized by an infiltration of several inflammatory cell populations. To explore aspects of the immune response in the pathogenesis of these bacteria we injected 10^6 CFU in footpads of BALB/c mice. After 1, 2, 3, 4, 7, 30 and 90 days immunohistochemistry was performed to compare presence and distribution of the inflammatory cytokines TNF-alpha, IL-1 beta, IL-6, IFN-gamma, IL-4, IL-10, and TGF-beta. Analysis of serial paraffin tissue sections showed strong participation and differences in distribution of cytokine-producing cells during the course of infection. Several TNF-alpha immunoreactive lymphocytes of the dermis were present during the course of the infection, but absent in the site of inflammation. During the first 4 days, IL-1 beta immunoreactivity was observed in dendritic epidermal cells and in cells surrounding the neutrophils around the grain. In later stages of infection, immunoreactive cells to this cytokine were mainly in the periphery of the microabscesses. Strong immunoreactivity was observed with IL-6 during the course of infection. Some cells in the epidermis and dermis, as well as muscle cells and several cells at the periphery of the microabscesses, showed strong IL-6 immunoreactivity. Cells immunoreactive to IL-4, IL-10, IFN-gamma and TGF-beta were present at the site of infection and, in later stages, in cells at the periphery of the microabscesses. In conclusion a mix of proinflammatory and antiinflammatory cytokines are produced at the same time by host cells. According to their distribution, inflammatory cytokines seems to have different functions during the course of infection with the intracellular bacterium *N. brasiliensis*.

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Key words: Nocardia, Cytokines, Intracellular bacteria, Immunocytochemistry

Introduction

Nocardia brasiliensis is a bacterium found as a saprophyte in soil. This intracellular facultative pathogen enters the skin by traumatic inoculation and causes actinomycetoma. The histopathology of the infection of *N. brasiliensis* in the mouse has been described at light and electron microscopy levels (Folb et al., 1976; Salinas-Carmona, 2000). There is progressive tissue destruction associated with an intense inflammatory response characterized by polymorphonuclear cells and macrophages, subsequently, lymphocytes accumulate progressively and several microabscesses are formed. This infection affects subcutaneous tissues and muscles; it can extend to adjacent bones and organs. Histopathology of human lesions closely resembles histopathological lesions observed in experimental mice (Salinas-Carmona, 2000; Guimaraes et al., 2003). Some of the specific participating cells and molecules have been characterized in actinomycetoma caused by *Streptomyces* and *Nocardia* (Conde et al., 1983; el Hassan et al., 2001; Guimaraes et al., 2003).

Host immune mechanisms and bacterial survival strategies are complex. In order to complete their replication cycle, bacteria modulate apoptotic pathways to control the lifespan of their host. At this point, cytokines play a main role. Inflammatory cytokines are produced by a variety of cells and may enhance growth of bacteria (Porat et al., 1991; Kanangat et al., 1999; Meduri et al., 1999). They are critically involved in the initiation and amplification of the local immune response and play a role in both protective immunity and the pathology of the infection (Van Crevel et al., 2002; Langhorne et al., 2004). TNF-alpha is a multifunctional

cytokine involved in various cell functions, such as metabolism, differentiation and mitosis. Besides, it controls angiogenesis, inflammation and the immune response. The relative amount of TNF-alpha at the site of infection determines whether the cytokine is protective or destructive (Bekker et al., 2000). IL-1beta is a highly proinflammatory cytokine, it upregulates host defences and functions as an immunoadjuvant (Dinarello, 1997). IL-6 is a pleiotropic cytokine that not only affects the immune system, but also acts in other biological systems and many physiological events in various organs. In a target cell, IL-6 can simultaneously generate functionally distinct or sometimes contradictory signals (Kamimura et al., 2003; Kishimoto, 2005a). IFN-gamma is an important proinflammatory cytokine in the host defence against viral and microbial infections; it induces a variety of physiologically significant responses that contribute to immunity (Shtrichman and Samuel, 2001). IL-4 is fundamental in the differentiation of Th2 cells, which promote humoral immunity (Li Weber and Krammer, 2003). IL-10 is an important suppressive cytokine, produced by a large number of immune cells; it is a key player in antiinflammatory immune responses focussed to minimize immunopathology (O'Garra et al., 2004). TGF-beta has bipolar functions in the immune system; it is well known for depressing the ongoing immune responses, but paradoxically, also initiates the host immune response by recruiting and activating immune cells, especially the Th17 lineage cells (Wahl et al., 2006; Wahl, 2007).

The regulation of the inflammatory response at the site of infection is thought to depend mainly on the release of cytokines that attract, focus and activate other inflammatory cells. Thus, the aim of this study is to study the presence and distribution of inflammatory cytokine-producing cells during the course of infection in an experimental model of actinomycetoma, as a medium to promote understanding of the interaction of *N. brasiliensis* with host defence mechanisms.

Materials and methods

Mice

9 to12-week-old BALB/c male and female animals were used. These animals are derived from a colony kindly donated by Carl Hansen from the Small Animals Section, Veterinary Resources Branch, National Institute of Health, Bethesda, MD. They were kept under standard conditions according to the Mexican Animal Protection Law (NOM-062-ZOO-1999).

Bacteria

Nocardia brasiliensis HUJEG-1 was isolated from a patient with actinomycetoma at the Hospital Universitario Dr. José E. González (Monterrey, México). This strain is maintained in our laboratory in a Sabouraud agar culture. It is registered as ATCC 700358.

Experimental infection

Infection was induced as previously described (Salinas-Carmona et al., 1999). Briefly, *N. brasiliensis* was cultured in brain heart infusion medium (Difco, Detroit, MI) to prepare a sterile saline suspension containing 10^7 CFU per ml in the log phase of growth. Mice were inoculated with 100 ml of this suspension (without adjuvant) in the footpad. After 1, 2, 3, 4, 7, 30 and 90 days mice were sacrificed (groups of 5 mice for each day) by cervical dislocation.

Immunocytochemistry

Tissue of the footpad was fixed in 4% formaldehyde in 0.2 M phosphate buffered saline pH 7.4 (PBS) overnight and embedded in paraffin. Serial sections of 7 µm-thickness were cut and mounted onto slides. Endogenous peroxidase was depleted with 0.3% hydrogen peroxide in methanol. After washing, immunocytochemistry was performed using polyclonal antibodies (Santa Cruz Biotechnology, Inc.) against mouse TNF-alpha (sc-1349 goat), mouse IL-1 beta (sc-1252 goat), mouse IL-6 (sc-1265 goat), mouse IFN-gamma (sc-9344 goat), mouse IL-4 (sc-1260 goat), mouse IL-10 (sc-1783 goat), and mouse TGF-beta (sc-146 goat). Sections were incubated overnight at 4°C with the various polyclonal antibodies, diluted at 1:500 PBS pH 7.4 with 0.5% Triton-X-100 (PBS-TX100). After washing in PBS-TX100, the sections were incubated for 2 h at room temperature with the secondary antiserum (polyclonal rabbit-anti-goat IgG, conjugated with horseradish peroxidase [HRP]; sc-2922, Santa Cruz Biotechnology, Inc.), in PBS-TX100. The HRP was visualized by means of 0.05% 3,3-diaminobenzidine tetrahydroxychloride (DAB, Sigma, USA) in PBS (pH 7.4) containing 0.01% hydrogen peroxide. Counterstaining was performed with haematoxylin.

Using serial sections, the serial areas with different cytokine staining were analyzed. Counts of immunoreactive cells were performed in five adjacent fields (x400) at the epidermis, inflammation site in the early infection, and in the microabscesses in later infection. In the dermis the cell counts were performed in five fields (x400) under the epidermis, avoiding the inflammation site (acute infection) and the microabscesses (chronic infection).

Negative controls included omission of the primary antibody, liquid-phase absorption with the homologous antigen (Santa Cruz Biotechnology, Inc.), and use of non-immune serum. Sections of the spleen, on the same slide, were also examined to act as positive controls.

Results

During the first four days, the inflammatory focus was mainly composed of neutrophils surrounding the bacteria. After four days, microabscesses were formed,

consisting of a grain of bacteria and an innermost zone of neutrophils, surrounded by lymphocytes and foamy macrophages. The microabscess was limited by a rim of fibroblastic cells and collagen fibres in a concentric orientation (Fig. 1). A line of mast cells was also present adjacent to this border.

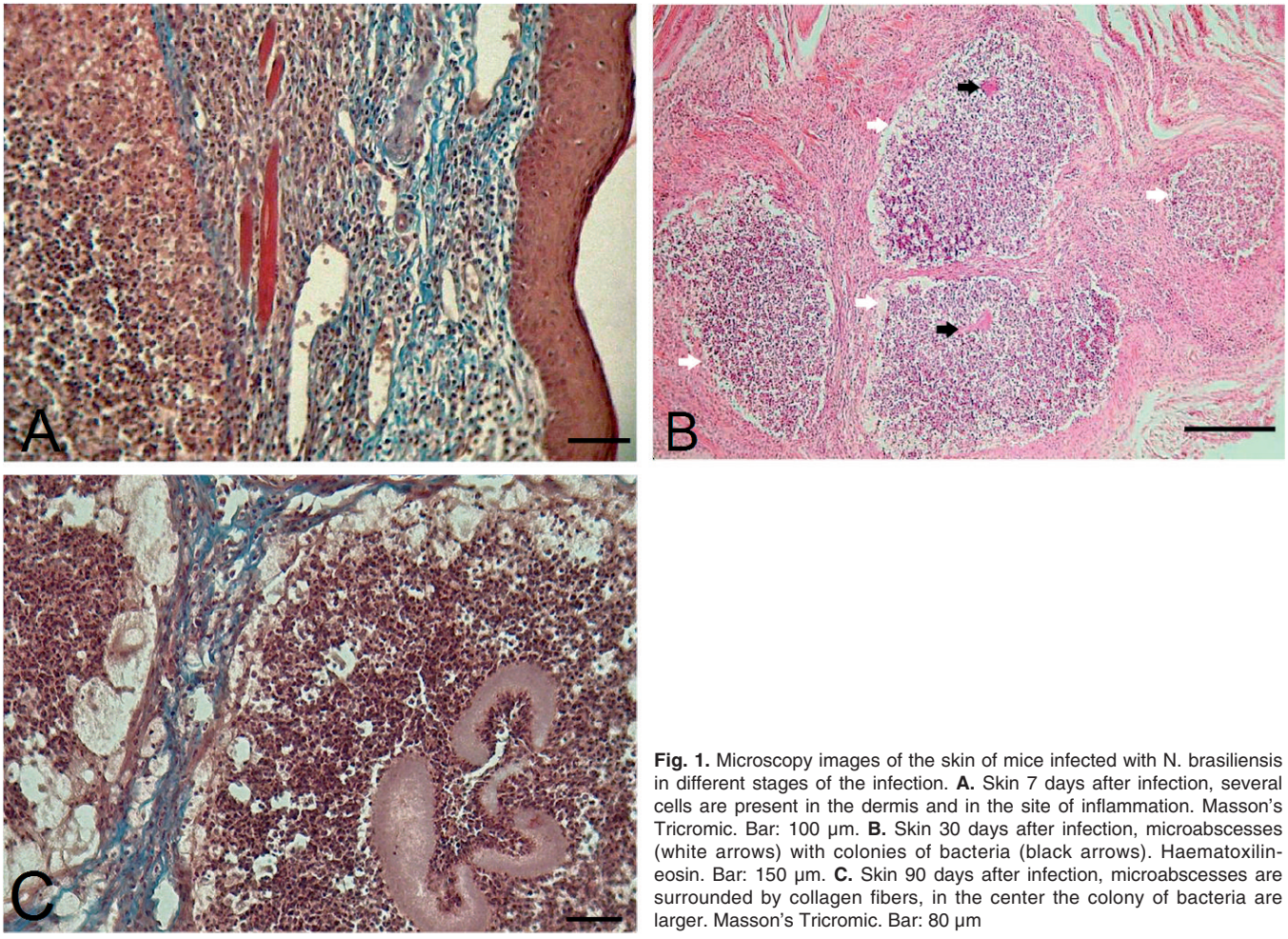

Fig. 1. Microscopy images of the skin of mice infected with N. brasiliensis in different stages of the infection. **A.** Skin 7 days after infection, several cells are present in the dermis and in the site of inflammation. Masson's Tricromic. Bar: 100 µm. **B.** Skin 30 days after infection, microabscesses (white arrows) with colonies of bacteria (black arrows). Haematoxilin-eosin. Bar: 150 µm. **C.** Skin 90 days after infection, microabscesses are surrounded by collagen fibers, in the center the colony of bacteria are larger. Masson's Tricromic. Bar: 80 µm

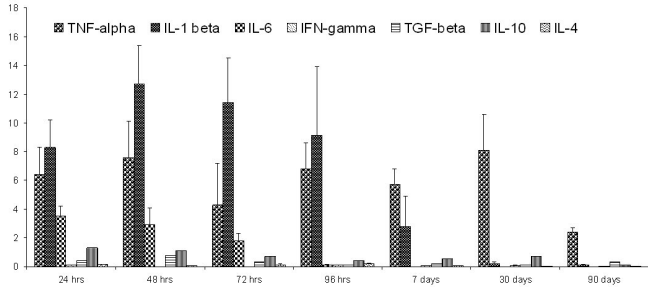

Fig. 2. Media and standard deviation of immunoreactive cells to inflammatory cytokines present at the epidermis during the course of infection with N. brasiliensis. Lymphocytes are immunoreactive to TNF-alpha. Langerhans cells are immunoreactive to IL-1 beta during the acute infection but no after 30 or 90 days postinfection. Lymphocytes are immunoreactive to IL-6 only during the first days of infection.

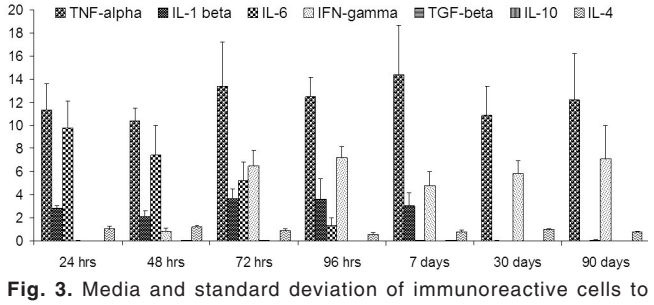

Fig. 3. Media and standard deviation of immunoreactive cells to inflammatory cytokines present at the dermis during the course of infection with N. brasiliensis. Lymphocytes are immunoreactive to TNF-alpha. Dendritic cells and some lymphocytes showed immunoreactivity to IL-1 beta. IL-6 immunoreactive lymphocytes were present only during the first days of infection. Lymphocytes and some macrophages showed immunoreactivity to IFN-gamma.

Analysis of cytokine-producing cells

Cytokine production in the early immune response against this bacterium was evident. Immunoreactive cells to all inflammatory cytokines were detected 24 hours after infection. Kinetics of cells producing inflammatory cytokines in epidermis, dermis, inflammatory zone, and, in later infection, in the periphery of microabscesses are shown in figures 2 to 4. During the course of the infection, intraepidermal lymphocytes and several cells of the dermis were immunoreactive to TNF-alpha (Fig. 5). No TNF-alpha immunoreactivity was observed at the site of inflammation, neither during early or late infection (microabscesses).

Dendritic cells (Langerhans cells) of the epidermis and some of the dermis showed immunoreactivity to IL-1 beta during the first days of infection (Fig. 6). These immunoreactive cells diminished after 7 and 30 days. During the first four days some cells immunoreactive to IL-1 beta were present in the inflammatory zone. After 30 and 90 days, macrophages in the periphery of the microabscesses were immunoreactive to IL-1 beta.

During the first four days, IL-6 immunoreactivity

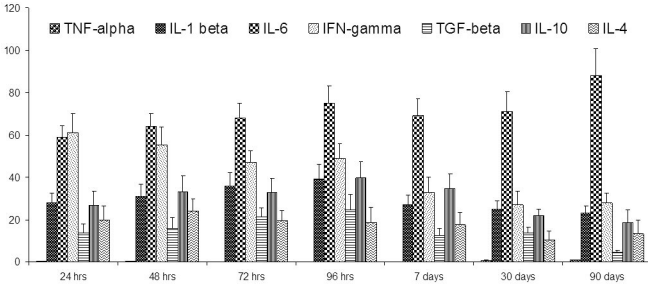

Fig.4. Media and standard deviation of immunoreactive cells to inflammatory cytokines present at the inflammation site (first days) and in the microabscesses during the course of infection with N. brasiliensis. Except for TNF-alpha, immunoreactive cells to all inflammatory cytokines are present.

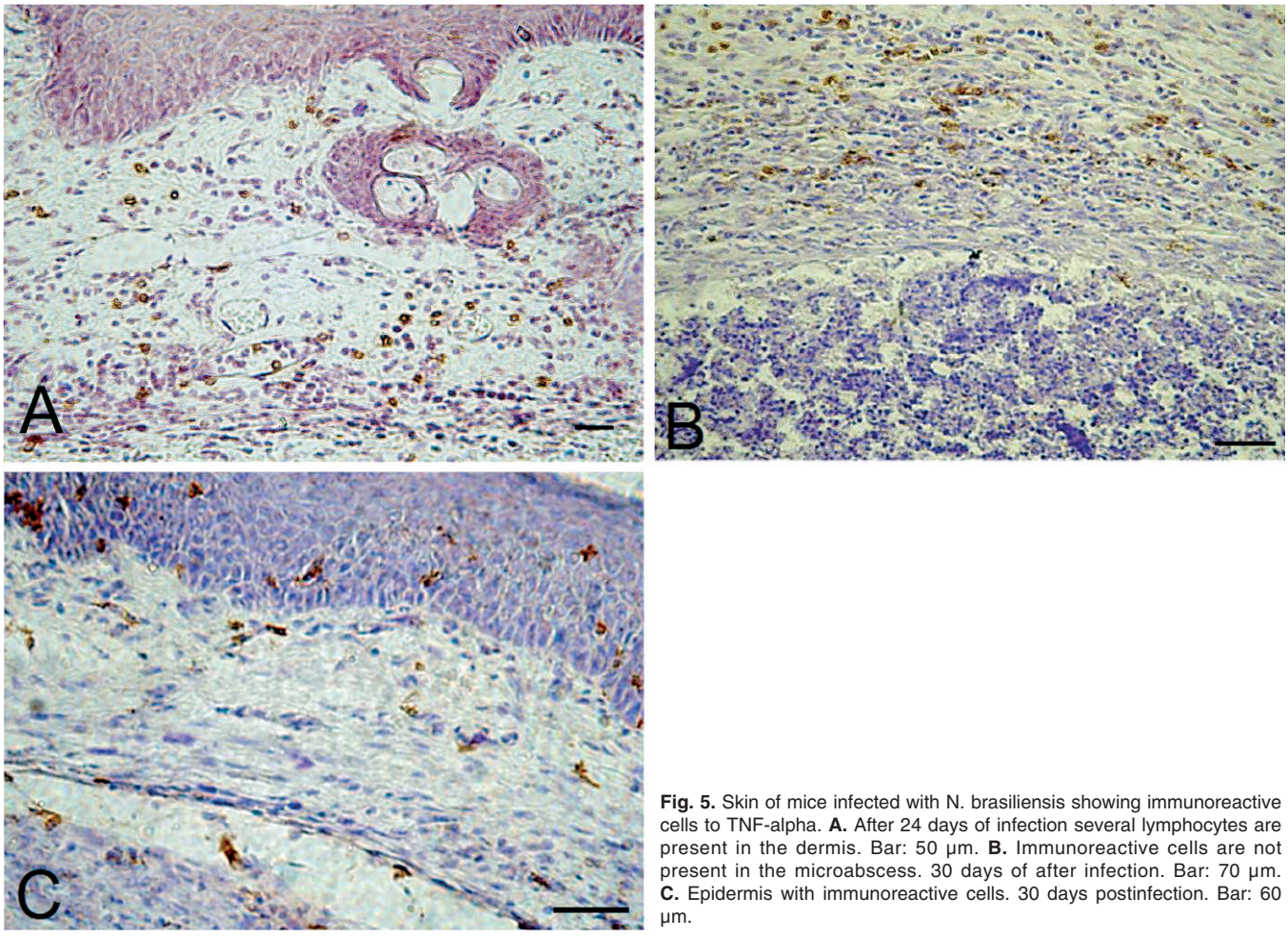

Fig. 5. Skin of mice infected with N. brasiliensis showing immunoreactive cells to TNF-alpha. **A.** After 24 days of infection several lymphocytes are present in the dermis. Bar: 50 µm. **B.** Immunoreactive cells are not present in the microabscess. 30 days of after infection. Bar: 70 µm. **C.** Epidermis with immunoreactive cells. 30 days postinfection. Bar: 60 µm.

was observed in epidermal lymphocytes, some dermal cells, and several cells at the inflammatory site. After 30 and 90 days, there were no immunoreactive cells in neither epidermis nor dermis, but several macrophages and lymphocytes cells showed immunoreactivity at the periphery of the microabscesses. Additionally, immunoreactivity was observed in skeletal muscle (Fig. 7).

IFN-gamma immunoreactivity was observed during the first days of the infection, mainly in cells at the inflammation site and later in the formed microbscesses (Fig. 8). Some dermal cells displayed IFN-gamma immunoreactivity.

During the course of infection, IL-4 immunoreactivity was observed in lymphocytes and a few macrophages close to the bacteria. Besides, a few immunoreactive lymphocytes were present in the dermis (Fig. 9).

During the first days, IL-10 immunoreactivity was observed in a few intraepidermal lymphocytes, as well as several cells in the inflammatory zone. When the microabscesses were formed, IL-10-producing cells were present in their periphery (Fig. 9B).

TGF-beta immunoreactivity was only observed in cells at the inflammatory zone during the course of infection (Fig. 9C).

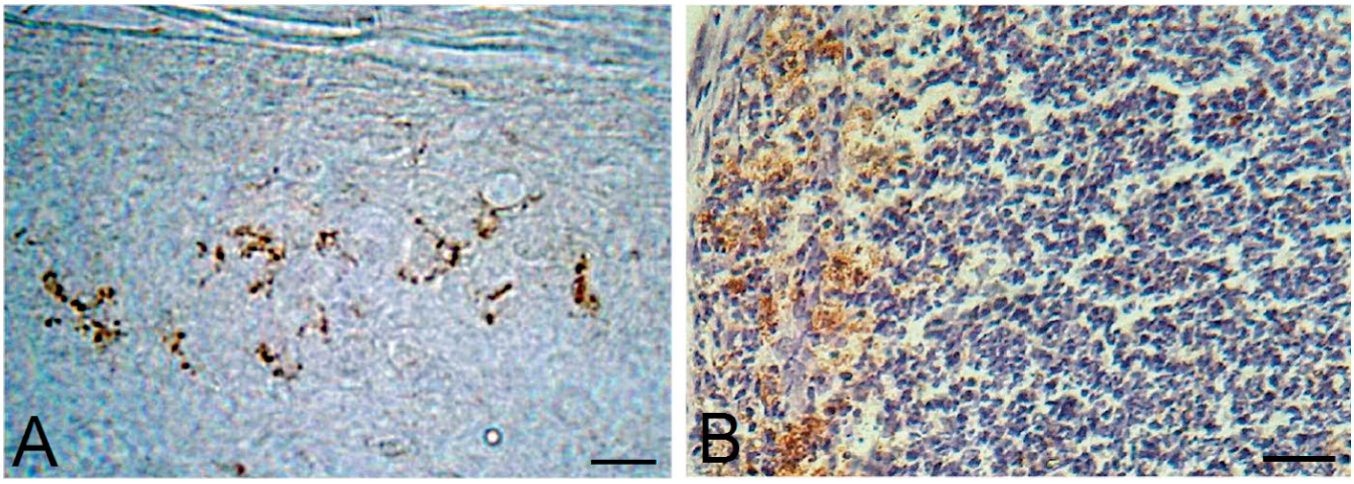

Fig. 6. Skin of mice infected with N. brasiliensis showing immunoreactive cells to IL-1 beta. **A.** After 48 hours of infection Langerhans cells are immunoreactive in the epidermis. Bar: 30 µm. **B.** After 30 days of infection immunoreactive cells are present in the periphery of the microabscess. Bar: 70 µm.

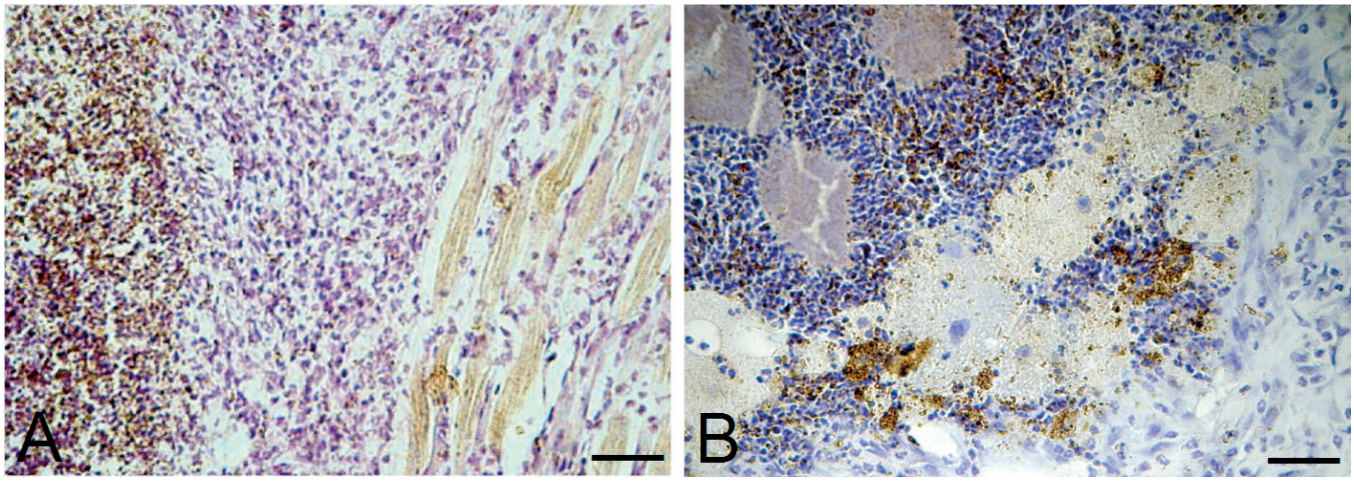

Fig. 7. Skin of mice infected with N. brasiliensis showing immunoreactive cells to IL-6. **A.** After 72 hours of infection immunoreactive cells are present in the site of inflammation in the dermis. Bar: 30 µm. **B.** After 30 days of infection immunoreactive cells are present in the periphery of the microabscess. Bar: 70 µm.

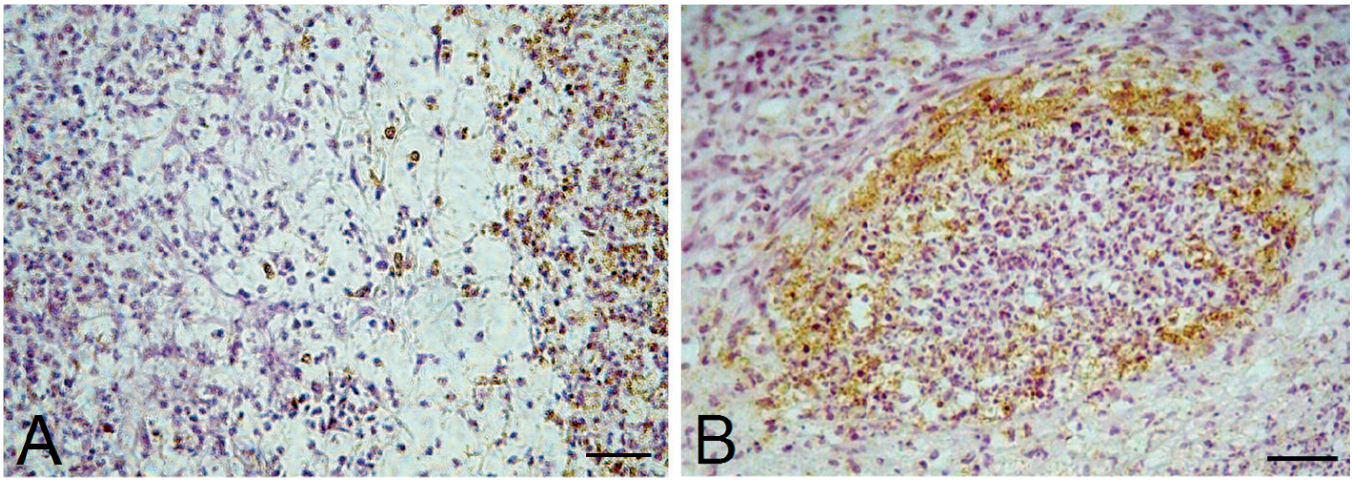

Fig. 8. Skin of mice infected with N. brasiliensis showing immunoreactive cells to IFN-gamma. **A.** After 4 days of infection immunoreactive cells are present in the site of infection and some disseminated in the dermis. Bar: 100 µm. **B.** After 7 days of infection immunoreactive cells are present in the periphery of the microabscess. Bar: 80 µm.

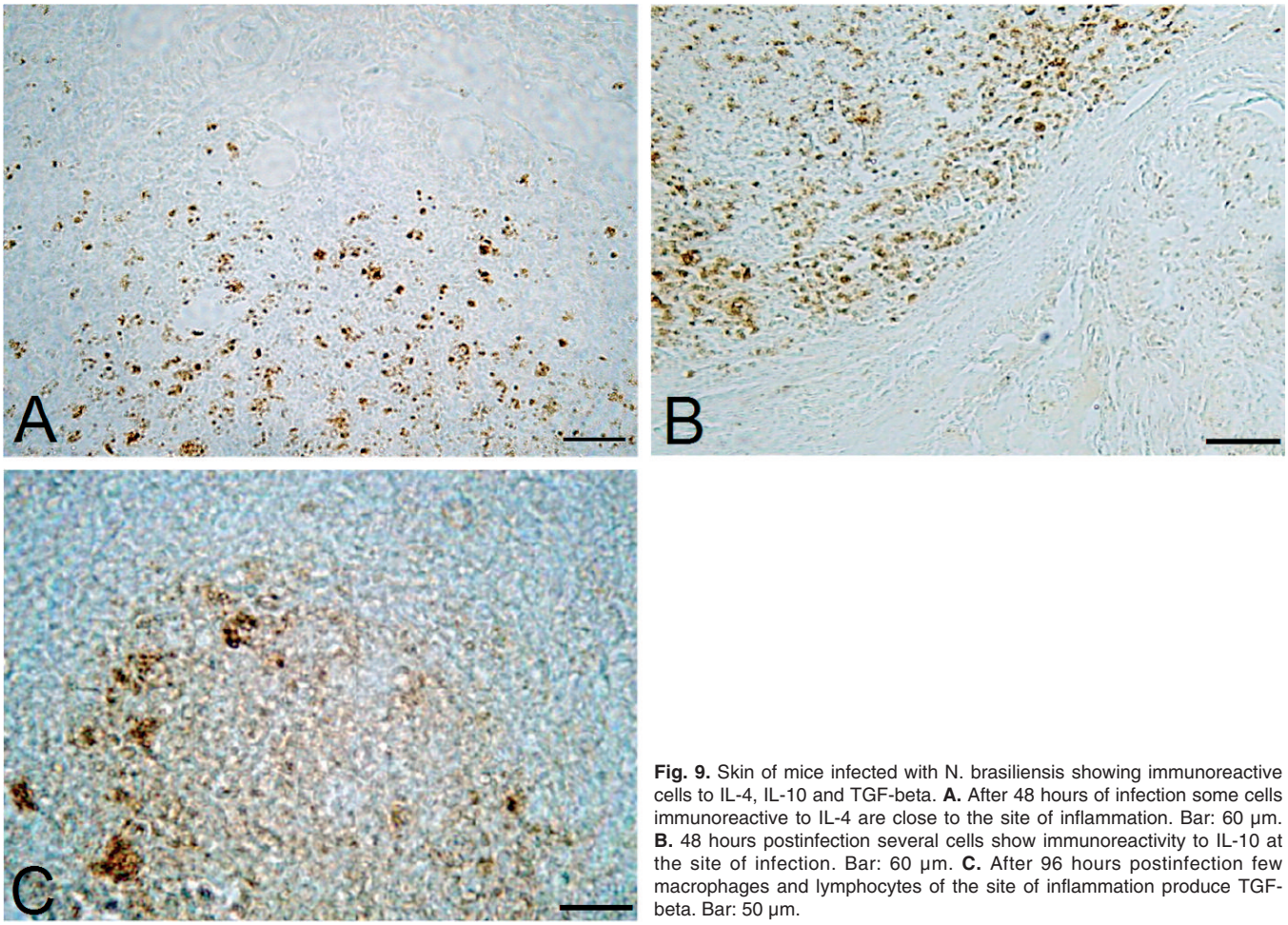

Fig. 9. Skin of mice infected with N. brasiliensis showing immunoreactive cells to IL-4, IL-10 and TGF-beta. **A.** After 48 hours of infection some cells immunoreactive to IL-4 are close to the site of inflammation. Bar: 60 µm. **B.** 48 hours postinfection several cells show immunoreactivity to IL-10 at the site of infection. Bar: 60 µm. **C.** After 96 hours postinfection few macrophages and lymphocytes of the site of inflammation produce TGF-beta. Bar: 50 µm.

Discussion

N. brasiliensis infection induces a strong immune response in BALB/c mice. This is demonstrated by the high number of cells producing inflammatory cytokines during the early and late stages of infection. The localization and type of cells that produce certain cytokines during the course of infection in this experimental model show a complex interaction between the host immune response and bacterial survival strategies.

Many aspects of antimicrobial host responses are orchestrated by a complex network of cytokines and their receptors (Wilson et al., 1998). Our findings on the distribution of the cells producing inflammatory cytokines show that these molecules play a central role as initiators, mediators and regulators of subcutaneous inflammation and subsequent pathological processes in skin tissues.

Resistance or susceptibility to intracellular bacteria is correlated with distinct patterns of cytokine production; some cytokine profiles are associated with bacterial clearance whereas other profiles may show disease progression (Yamamura et al., 1991; Zhang and Tarleton, 1996; Bekker et al., 2000). Our results on the localization of the inflammatory cytokines IL-1beta, IL-6 and IFN-gamma, present in the epidermis and dermis during early infection and later especially at the periphery of the formed microabcesses suggest a participation of these cytokines in the control of bacterial multiplication. Similar results have been reported with *Trypanosoma cruzi* (Magalhaes-Santos and Andrade, 2005).

It is interesting that, throughout the course of the infection, only epidermal and dermal lymphocytes expressed TNF-alpha, whereas no TNF-alpha expression was observed at the inflammation focus. The importance of TNF-alpha in microabscess formation might be mainly reflected in its induction of endothelial alterations such as increase in vascular permeability and induction of the expression of adhesion molecules that facilitates the binding of leukocytes and their subsequent diapedesis (Kasahara et al., 1989; Kindler et al., 1989; Senaldi et al., 1996).

We found that dendritic cells, among other cells, produce IL-1 beta only during the first days of infection, these results are consistent with other reports, stating that Langerhans cell-derived IL-1 beta plays an essential role in the initiation of primary immune responses in skin (Enk et al., 1993).

In this study, IL-6 strongly participated. Immunoreactive cells to this cytokine were observed in the periphery of the microabscesses, mainly later in infection, IL-6 was originally known as B cell stimulatory factor, but has been proven to be a cytokine with multiple biological activities during inflammation and the immune response (Kishimoto, 2005a,b). As has been reported for *E. coli* neumonia (Jones et al., 2006), IL-6 might enhance polymorphonuclear recruitment, activate STAT transcription factors, and decrease bacterial burdens. During the first days of infection several cells of the epidermis and dermis showed IL-6 immunoreactivity, perhaps in this stage they participate in the release of histamine from mast cells (Kikuchi et al., 2002). On the other hand, the presence of IL-6 in skeletal muscle might be due to cellular damage, as has been reported previously (Jonsdottir et al., 2000; Pedersen et al., 2001).

IFN-gamma-producing cells are present close to the inoculated bacteria, during early infection, whereas macrophages and lymphocytes in the periphery of the microabscess display IFN-gamma immunoreactivity later on. Perhaps, besides activating macrophages, this cytokine is modulating the formation of the microabscess as has been suggested with the granuloma caused by *Mycobacterium tuberculosis* (Fuller et al., 2003)

It is known that IL-4 is a potent activator of Th2 response, where IL-10 is produced to diminish Th1 response. In this study, IL-4 and IL-10 were present in cells close to the bacteria during all the course of infection with *N. brasiliensis*. It is difficult to say that IL-4 is produced to induce a Th2 response, because also IFN-gamma is produced, and infections induce the expansion of interleukin-10-producing regulatory cells (Mege et al., 2006). On the other hand, IL-4 seems to be a good target for bacterial regulation for its own benefit, because the expression of the gene of IL-4 can be controlled by several factors (Lavender et al., 2000; Li-Weber et al., 1997). It should be noted that *N. brasiliensis* secretes substances like brasilicardin A and brasilinolide A, which are potent immunosuppresive factors for lymphocytes, even stronger than known immunosuppressive agents like cyclosporine A (Shigemori et al., 1998).

TGF-beta expression was observed at the inflammation site throughout the infection which is consistent with its roles as both an initiator of the host response and an inhibitor of ongoing immune responses (Wahl, 2007). This multifunctional cytokine might also be involved in the formation of a collagen capsule around the microabscess as it has been reported to be a key regulator of extracellular matrix degradation and synthesis (Verrecchia and Mauviel, 2002).

The determination of a cytokine-producing phenotype is dependent on several factors, such as age, site of initial antigen exposure, bacterial dose, genetic constitution, health status, etc. (Seder and Paul, 1994; Howard and Zwilling, 1998; Kasuga-Aoki et al., 1999; Adkins et al., 2000; Hessle et al., 2005; Peruhype-Magalhaes et al., 2005). In this study, we showed that dendritic cells, small mononuclear round cells (lymphocytes), big round cells (macrophages) and cylindrical muscle cells produce different cytokines, although some cells seem to produce several cytokines. Further studies of co-localization are in process to elucidate this.

In summary, inflammatory cytokines, synthesized by

different cell types, are strongly expressed during the course of infection by the intracellular bacterium *N. brasiliensis*. The pattern of cells immunoreactive to these cytokines showed differences in distribution and number during the infection. *N. brasiliensis*, however, is able to survive the host's strong immune response. It seems that this bacterium can manage the host defence reaction towards its own growth advantage and survive, may be by secreting factors that modulate the bouquet of regulatory cytokines.

Acknowledgements. The authors express their gratitude to Luz Isabel Perez Rivera and Patricia Alejandra Gallegos Velasco, for their help in infecting the mice, and to Maria de la Luz Elizondo Cisneros for technical support. This work was supported by grants from PROMEP (SEP) 103.5/05/2485 and PAICYT SA1420-06 (UANL).

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Accepted November 21, 2007