The Journal of Immunology

RESEARCH ARTICLE | JULY 15 2002

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J Immunol (2002) 169 (2): 898–905.
https://doi.org/10.4049/jimmunol.169.2.898

Inhibition of the Spontaneous Apoptosis of Neutrophil Granulocytes by the Intracellular Parasite *Leishmania major*[1]

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Macrophages are the major target cell population of the obligate intracellular parasites *Leishmania*. Although polymorphonuclear neutrophil granulocytes (PMN) are able to internalize *Leishmania* promastigotes, these cells have not been considered to date as host cells for the parasites, primarily due to their short life span. In vitro coincubation experiments were conducted to investigate whether *Leishmania* can modify the spontaneous apoptosis of human PMN. Coincubation of PMN with *Leishmania major* promastigotes resulted in a significant decrease in the ratio of apoptotic neutrophils as detected by morphological analysis of cell nuclei, TUNEL assay, gel electrophoresis of low m.w. DNA fragments, and annexin V staining. The observed antiapoptotic effect was found to be associated with a significant reduction of caspase-3 activity in PMN. The inhibition of PMN apoptosis depended on viable parasites because killed *Leishmania* or a lysate of the parasites did not have antiapoptotic effect. *L. major* did not block, but rather delayed the programmed cell death of neutrophils by ~24 h. The antiapoptotic effect of the parasites could not be transferred by the supernatants, despite secretion of IL-8 by PMN upon coculture with *L. major*. In vivo, intact parasites were found intracellularly in PMN collected from the skin of mice 3 days after s.c. infection. This finding strongly suggests that infection with *Leishmania* prolongs the survival time of neutrophils also in vivo. These data indicate that *Leishmania* induce an increased survival of neutrophil granulocytes both in vitro and in vivo. *The Journal of Immunology*, 2002, 169: 898–905.

Leishmaniasis is caused by the infection with protozoa of the genus *Leishmania*. In the mammalian host *Leishmania* are obligate intracellular pathogens; most of them are rapidly killed in the extracellular tissue environment. After being internalized by phagocytes, they can escape the toxic extracellular milieu and survive intracellularly (for review, see Ref. 1). The primary host cells of the intracellular parasites are macrophages, but fibroblasts can also harbor *Leishmania* in the chronic latent phase of infection (2).

Immediately after infection with *Leishmania major* in the skin, a local inflammatory process is initiated, which involves the accumulation of leukocytes. In the earliest wave of infiltration, polymorphonuclear neutrophil granulocytes (PMN)[3] are recruited within the first hours after infection, followed by monocytes/macrophages 2–3 days later (3, 4). Although the uptake and intracellular survival of *Leishmania* in macrophages are well established, little is known about the role of PMN as host cells for the parasites.

PMN play a vital role by phagocytosing and destroying microorganisms such as bacteria and fungi. Neutrophils were reported to phagocytose also *Leishmania* promastigotes (5) using both opsonin-dependent and opsonin-independent uptake mechanisms (6). *Leishmania* were reported to interfere with the production of reactive oxygen intermediates by neutrophils (7, 8). Consequently, not all internalized parasites are killed immediately in PMN, as demonstrated by the finding that morphologically intact parasites were detected in granulocytes of infected animals (9). Moreover, infected neutrophils were observed in chronic lesions of mice after infection with *L. major* (10). Therefore, after being recruited to the site of infection, granulocytes can internalize *Leishmania* and possibly can provide an intracellular milieu for the survival of the parasites.

Neutrophil granulocytes are inherently short-lived cells with a $t_{1/2}$ of only ~6–10 h in the circulation, after which they undergo apoptosis (11, 12). It is an active and well-regulated process that is characterized by specific phenomena such as cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation, membrane blebbing, and, finally, the decay into apoptotic bodies (12, 13). Although apoptosis is an intrinsic cell process, the life span of mature neutrophils can be extended in vitro by incubation with either proinflammatory cytokines including GM-CSF, G-CSF (14), IL-8 (15), IL-1β (16), glucocorticoids (17), or bacterial products such as LPS and fMLP (16, 18).

Moreover, several microbial pathogens have been reported to influence this process as common strategy to overcome host defenses (for review, see Ref. 19). Thus, apoptosis induction in cells of the innate or adaptive immunity appears to be a way to escape antimicrobial defense of the host. Microbial pathogens such as *Escherichia coli* (20) and *Candida albicans* (21) were found to induce apoptosis of PMN. Induction of apoptotic death of both mononuclear and polymorphonuclear leukocytes by *Fusobacterium nucleatum* was suggested to mediate immunosuppression in periodontal disease (22). In contrast, inhibition of apoptosis of host cells by intracellular pathogens such as *Theileria* (23), *Toxoplasma* (24), or the agents of human granulocytic ehrlichiosis (HGE) (25) can provide an intracellular niche for the pathogens by extending the life span of the host cells.

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Received for publication August 20, 2001. Accepted for publication April 17, 2002.

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[1] This work was supported by the Deutsche Forschungsgemeinschaft (SFB 367/B10, GRK 288/B8).

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[3] Abbreviations used in this paper: PMN, polymorphonuclear neutrophil granulocyte; AMC, 7-amino-4-methylcoumarin; HGE, human granulocytic ehrlichiosis; PS, phosphatidylserine.

0022-1767/02/$02.00

In the present study, we investigated whether also *Leishmania* can prolong the lifetime of neutrophils to secure an intracellular environment for its survival. We found that coincubation with *L. major* promastigotes results in a delay of neutrophil apoptosis, which is associated with a decrease in caspase-3 activity.

Materials and Methods

Isolation of human peripheral blood granulocytes

Peripheral blood was collected by venipuncture from healthy adult volunteers using lithium-heparin, which was reported to be the optimal anticoagulant for preserving granulocyte viability (26). Blood was layered on Histopaque 1119 (Sigma-Aldrich, Deisenhofen, Germany) and centrifuged for 20 min at 800 × *g*. The plasma and the interphase consisting mainly of lymphocytes and monocytes were discarded. The granulocyte-rich layer below the interphase was collected, leaving the erythrocyte pellet on the bottom of the tube. Granulocytes were washed twice in RPMI 1640 medium (Seromed-Biochrom, Berlin, Germany), and further fractionated on a discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient consisting of layers with densities of 1105 g/ml (85%), 1100 g/ml (80%), 1093 g/ml (75%), 1087 g/ml (70%), and 1081 g/ml (65%). After centrifugation for 20 min at 800 × *g*, the interface between the 80% and 85% Percoll layers was collected and washed twice in RPMI 1640 medium. All procedures were conducted at room temperature. The preparations contained >99% granulocytes, of which >95% were neutrophils and 1–4% were eosinophils, as determined by Giemsa staining of cytocentrifuged (Shandon, Pittsburgh, PA) samples. Eosinophil granulocytes were identified as cells containing numerous small orange-red-stained intracellular granula. Cell viability was >98%, as determined by trypan blue exclusion.

Leishmania *and coincubation experiments*

The origin and propagation of the cloned virulent line of *L. major* (MHOM/IL/81/FEBNI) have been described elsewhere (27). Stationary phase promastigotes were collected from in vitro cultures in biphasic NNN blood agar medium. PMN were coincubated at 37°C in a volume of 1 ml RPMI 1640 medium (Life Technologies Laboratories, Eggenstein, Germany) supplemented with 50 μM 2-ME, 2 mM L-glutamine, 10 mM HEPES, 100 μg/ml penicillin, and 160 μg/ml gentamicin (all Seromed-Biochrom), and with 10% FCS (Life Technologies Laboratories) with *L. major* promastigotes at a parasite-PMN ratio of 5:1 in a humidified atmosphere containing 5% CO_2.

Coincubation experiments were also conducted by using killed promastigotes or parasite lysates. *L. major* promastigotes were killed by treatment with ethanol, as described (28). Briefly, stationary phase promastigotes at a concentration of 1×10^7/ml were incubated in PBS containing 30% ethanol for 30 min at room temperature, followed by washing twice in PBS. Parasite lysates were obtained by freezing and thawing *L. major* promastigotes (1×10^7/ml in PBS) five times. In the coincubation experiments, killed *Leishmania* and parasite lysate were used at a parasite-PMN ratio of 5:1.

To assay the antiapoptotic effect of the supernatants from *L. major*-PMN cocultures, neutrophils were coincubated with *L. major* promastigotes for 24 h, as described above. Supernatants were collected and freshly isolated, and pelleted PMN were resuspended in these supernatants (supernatant concentration = 100%) for 24 h.

Assessment of PMN apoptosis

Morphological assessment of apoptosis. In neutrophils, morphological changes of apoptosis are striking and include separation of nuclear lobes and darkly stained pyknotic nuclei (12, 13). Accordingly, the morphological criteria for neutrophil apoptosis were one or more densely stained nuclear fragments and the absence of chromatin within nuclear lobes/fragments. Nuclear morphology was assessed on Giemsa-stained cytocentrifuge slides. Cell morphology was examined under oil immersion light microscopy, and a minimum of 200 cells/slide was examined and graded as apoptotic/nonapoptotic.

Annexin V binding

Annexin V exhibits calcium-dependent binding to phosphatidylserine (PS) expressed in the outer membrane leaflet of apoptotic PMN (29). Labeling of apoptotic cells with annexin V FITC (Roche Molecular Biologicals, Mannheim, Germany) was performed, as recommended by the manufacturer. Labeled cells were analyzed by flow cytometry using a FACSCalibur with CellQuest software (BD Biosciences, San Diego, CA).

TUNEL assay of chromatin fragmentation

The TUNEL assay (In Situ Cell Death Detection kit; Roche Molecular Biologicals) was used to detect apoptotic cell death by enzymatic labeling of DNA strand breaks with fluorescein-dUTP and TdT (30). Briefly, cytocentrifuge slides containing 1×10^5 cells were fixed in 4% formaldehyde/PBS (pH 7.4) for 60 min at room temperature, washed in PBS, and then suspended in permeabilization solution (0.1% Triton X-100/0.1% sodium citrate) for 3 min on ice. Cells were washed again, resuspended in 50 μl TUNEL-reaction mixture or in 50 μl label solution alone (negative control), and incubated in a humidified dark chamber at 37°C, followed by washing in PBS. The green fluorescence of apoptotic nuclei was detected by fluorescence microscopy.

Gel electrophoresis of low m.w. DNA

Internucleosomal DNA fragmentation was assessed using the ApopLadder Ex kit (Takara Biomedicals, Takara Shuzu, Otsu, Shiga, Japan). Briefly, neutrophil granulocytes were incubated with or without *L. major* promastigotes, and the fragmented DNA was separated from intact chromatin of 1×10^6 granulocytes. Proteins and RNA were eliminated by enzymatic digestion. Low m.w. DNA was precipitated with ethanol and analyzed in 1.8% agarose gels.

Fluorometric analysis of caspase-3 activity

Neutrophil granulocytes were incubated with/without *L. major* promastigotes, as described above. A total of 1×10^6 cells was counted and collected by centrifugation and resuspended in 100 μl lysis buffer (100 mM HEPES, pH 7.5, 10% sucrose, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 1 mM EDTA, and 1 mM PMSF). Cell lysate was incubated with 12 μM fluorogenic peptide substrate DEVD-AMC (7-amino-4-methylcoumarin; Biomol, Hamburg, Germany) in a 96-well microtiter plate (Greiner, Frickenhausen, Germany) at room temperature. The cleavage of DEVD-AMC was monitored by AMC (Sigma-Aldrich) liberation in CytoFluor 2350 plate reader (Millipore, Bedford, MA) using 360-nm excitation and 460-nm emission wavelength. Fluorescence was measured every 2 min during a 60-min period, and fluorescence units were converted to pmol amounts of AMC using a standard curve generated with free AMC. To assess the specificity of the reaction, the competitive inhibitor of caspase-3 DEVD-CHO (50 nM) was added to the samples. This treatment blocked DEVD-AMC cleavage (not shown).

SYTO-16/annexin V double staining to visualize simultaneously viable parasites and apoptotic PMN

Neutrophil granulocytes were coincubated with *L. major* promastigotes at a parasite-PMN ratio of 5:1 at 37°C in a humidified atmosphere containing 5% CO_2 in a volume of 1 ml RPMI 1640 medium supplemented with 10% FCS (see above). After 18 h, cells were stained with SYTO-16 (Molecular Probes, Leiden, The Netherlands) and annexin V-Alexa 568. Staining with 5 μmol SYTO-16 for 10 min at room temperature was used to visualize viable intracellular parasites in PMN. This dye penetrates cell membranes and stains DNA. SYTO-16 stains, therefore, nuclei and kinetoplast of viable cells/parasites green. SYTO-16 has been reported to be useful to visualize also apoptotic nuclei in live cells (31). Subsequent to the SYTO-16 staining, the cells were stained with annexin V to reveal apoptotic cells, as described above.

Cytokine assays

Neutrophil granulocytes were coincubated with *L. major* promastigotes at a parasite-PMN ratio of 5:1 at 37°C in a humidified atmosphere containing 5% CO_2 in a volume of 1 ml RPMI 1640 medium supplemented with 10% FCS (see above). Culture supernatants were collected after 18 and 24 h and stored at −20°C until cytokine determination. IL-8, GM-CSF, and TNF-α were measured using ELISA (R&D Systems, Wiesbaden, Germany), according to the manufacturer's instructions.

Subcutaneous air-pouch technique

Female BALB/c mice were obtained from Charles River Breeding Laboratories (Sulzfeld, Germany) and were used at 8–12 wk of age. Air pouches were raised on the dorsum by s.c. injections of 4 ml sterile air on days 0 and 3, as previously described (32). On day 6, 1×10^7 *L. major* promastigotes in 1 ml PBS were injected into the air pouch. As control, 10^7 latex beads (3 μm diameter; Sigma-Aldrich) in 1 ml PBS or as a sterile inflammatory agent 0.5% oyster type II glycogen in 1 ml PBS (Sigma-Aldrich) (33) were injected into the pouch. Three days after infection, the mice were killed and 1×10^5 exudate cells were centrifuged on microscope slides at 200 × g for

5 min using a cytocentrifuge. The slides were air dried, fixed with methanol, and then stained with Giemsa.

Results

Coincubation with L. major *promastigotes inhibits neutrophil apoptosis in vitro*

Morphological assessment of PMN apoptosis. Neutrophils undergo constitutive apoptosis when aging in vitro. Aging granulocytes exhibit classical features of apoptosis, such as cell shrinkage, cytoplasmic condensation, and condensation of nuclear heterochromatin (11–13). Accordingly, neutrophil apoptosis can be assessed by various parameters, including changes in cellular morphology. Using these criteria, the ratio of apoptotic cells was determined in highly purified granulocytes (purity >99%) cultured in vitro in the absence or presence of *L. major* promastigotes. The majority of neutrophil granulocytes (63 ± 11%, $n = 8$, range of 50–80%) were found to have an apoptotic nuclear morphology after incubation in vitro for 24 h (Figs. 1*B* and 2). The rate of apoptosis was strongly reduced when neutrophils were coincubated with promastigotes of *L. major*; 30 ± 6% of the cells ($n = 8$, range of 15–45%) had apoptotic morphology after 24 h of coincubation (Figs. 1*A* and 2). The ratio of infected PMN was 74 ± 9%. Infected granulocytes contained at average 2.5 ± 0.5 intracellular parasites in a range of one to seven parasites/cell. After 24 h of coincubation with *L. major*, most of the apoptotic PMN were those without intracellular parasites. However, a small proportion (5.5 ± 3.3%) of infected granulocytes also had apoptotic nuclear morphology.

Leishmania protected neutrophils from apoptosis without evidence of significant necrotic death, as assessed by trypan blue exclusion (not shown).

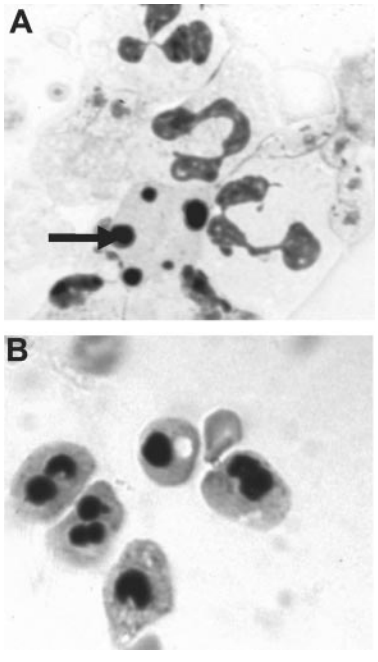

FIGURE 1. Morphological appearance of human PMN incubated in the presence (*A*) and absence (*B*) of viable *L. major* promastigotes. PMN were cultured at 5×10^6/ml in RPMI 1640 medium containing 10% FCS for 24 h. Cytocentrifuge preparations were stained with Giemsa. *A*, In the presence of *L. major* (PMN-*L. major* ratio 1:5), only a minor population of cells shows apoptotic morphology (arrow). *B*, PMN incubated with medium alone exhibit morphological features of apoptosis.

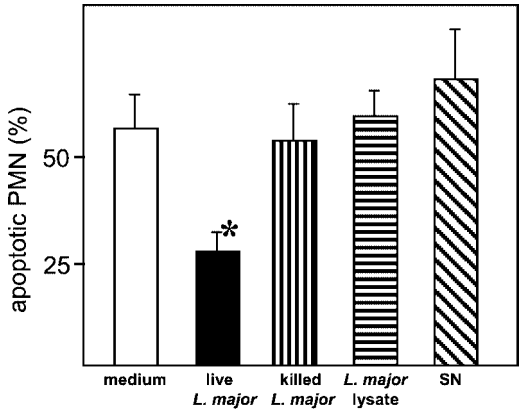

FIGURE 2. PMN apoptosis in the presence of viable or killed *L. major* promastigotes. Neutrophils were cultured at 5×10^6/ml in RPMI 1640 medium containing 10% FCS for 24 h in the presence of viable or killed *L. major* promastigotes, with a lysate of *L. major* promastigotes or with supernatants of PMN-*L. major* cocultures. The PMN-*L. major* ratio was 1:5. The percentage of apoptotic cells was determined by microscopical evaluation of >200 cells on cytocentrifuge preparations stained with Giemsa. The data shown are from three independent experiments.

Detection of nuclear DNA nicks using the TUNEL staining

In addition to the analysis of apoptotic nuclear morphology, the inhibitory effect of *L. major* on neutrophil apoptosis was investigated by the TUNEL assay, which reveals the apoptotic fragmentation of nuclear DNA. After incubation in vitro for 24 h, most of the neutrophils showed an intensive TUNEL staining (Fig. 3*B*). The majority of neutrophils that were coincubated with *L. major* promastigotes remained TUNEL negative (Fig. 3*A*).

Assessment of PMN apoptosis by detection of histone-associated low m.w. DNA

Electrophoresis of extracted low m.w. DNA from PMN populations aged in vitro for 24 h showed a ladder pattern of internucleosomal cleavage indicative of endonuclease activation. The analysis of internucleosomal DNA degradation revealed an inhibition of apoptosis in PMN by *L. major*, as shown by the marked

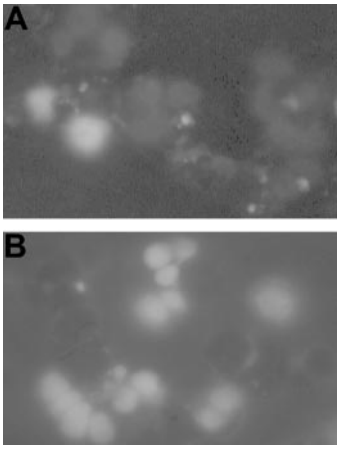

FIGURE 3. PMN apoptosis detected by TUNEL assay. PMN were cultured at 5×10^6/ml in RPMI 1640 medium containing 10% FCS for 24 h. Cytocentrifuge preparations were subjected to TUNEL staining, as described in *Materials and Methods*. *A*, In the presence of *L. major* (PMN-*L. major* ratio 1:5), most PMN remain TUNEL negative, and only few cells are TUNEL positive. *B*, The majority of PMN incubated in the absence of *L. major* show intensive TUNEL staining.

reduction of low m.w. chromatin fragments after coincubation with the parasites as compared with neutrophils incubated in medium alone (Fig. 4).

Annexin V staining of PS on the neutrophil outer membrane

Visible changes of nuclear morphology are associated with progressed stage of cellular apoptosis. Similarly, TUNEL positivity and the appearance of typical apoptotic low molecular DNA ladder are associated with the later stage of the apoptotic process. An earlier marker of PMN apoptosis is the appearance of PS on the outer membrane, a process that is called membrane flip. PS can be detected by staining with annexin V. Annexin V staining revealed high PS expression in the majority of neutrophils after incubation in vitro for 18 h (Fig. 5). Coincubation with *L. major* resulted in a marked decrease in annexin V binding (Fig. 5).

Phagocytosis of viable parasites is a strong inducer of delayed neutrophil apoptosis

The spontaneous apoptosis was inhibited only in the presence of viable *L. major* promastigotes. Coincubation of neutrophils with killed promastigotes or a lysate of parasites did not lead to a decrease in the rate of apoptotic cells after 24 h of in vitro culture (Fig. 2). The antiapoptotic activity of *Leishmania* did not strictly depend on the phagocytosis of the parasites because some neutrophils without intracellular *Leishmania* also showed a nonapoptotic nuclear morphology after 24 h of coincubation with *L. major*. The uptake of *Leishmania* appeared to be a major factor of delayed apoptosis because microscopical examination of Giemsa-stained cytocentrifuge preparates, as shown above, revealed only a minor population ($5.5 \pm 3.3\%$) of infected neutrophils that was apoptotic. To confirm this observation, a double staining was applied to visualize both live parasites and apoptotic neutrophils. Neutrophils were coincubated with *L. major* for 18 h and then stained with the live stain SYTO-16 to visualize viable cells. The cells were then stained with annexin V PE to detect apoptotic cells. The majority

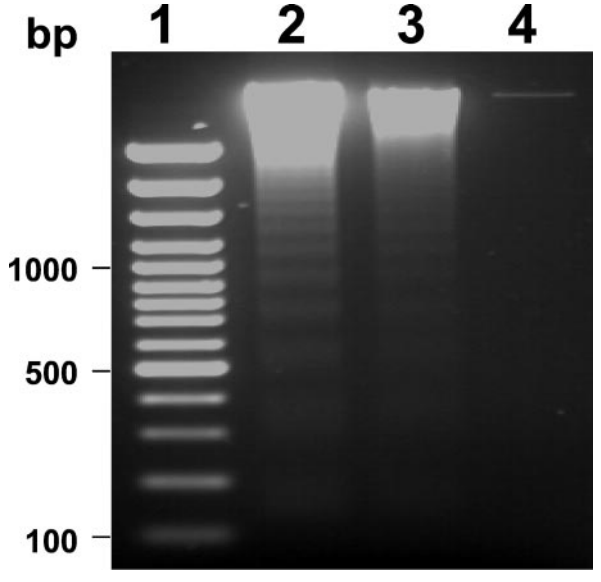

FIGURE 4. Chromatin fragmentation in human PMN measured by internucleosomal DNA degradation. Agarose gel electrophoresis patterns of low m.w. DNA extracted from freshly isolated neutrophils (*lane 4*) and from neutrophils cultured for 18 h at 5×10^6/ml in RPMI 1640 medium containing 10% FCS with (*lane 3*) or without *L. major* promastigotes (*lane 2*) at a PMN-*L. major* ratio of 1:5. *Lane 1*, Shows 100-bp molecular size marker.

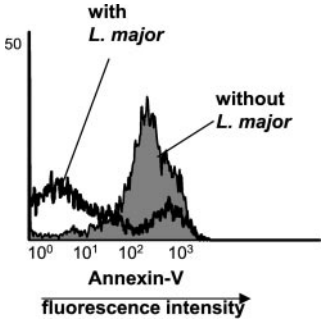

FIGURE 5. Flow cytometry profiles of PMN stained with annexin V. Neutrophils were incubated for 18 h at a concentration of 5×10^6/ml in RPMI 1640 medium containing 10% FCS without or with *L. major* promastigotes at a PMN-*L. major* ratio of 1:5 before staining with annexin V FITC. The *x*-axis shows the green fluorescence intensity (FL-1) of cells stained with annexin V FITC. The numbers indicate the ratio of annexin V-positive cells.

(>95%) of neutrophils that contained green-stained *Leishmania* were nonapoptotic viable cells, as detected by the positive staining with SYTO-16 and lack of reactivity with annexin V (as shown in Fig. 6). Almost none of the red-stained apoptotic neutrophils harbored *Leishmania* (Fig. 6). However, a minor population (2%) of apoptotic neutrophils was infected (not shown). These findings suggest that the internalization of living *Leishmania* promastigotes

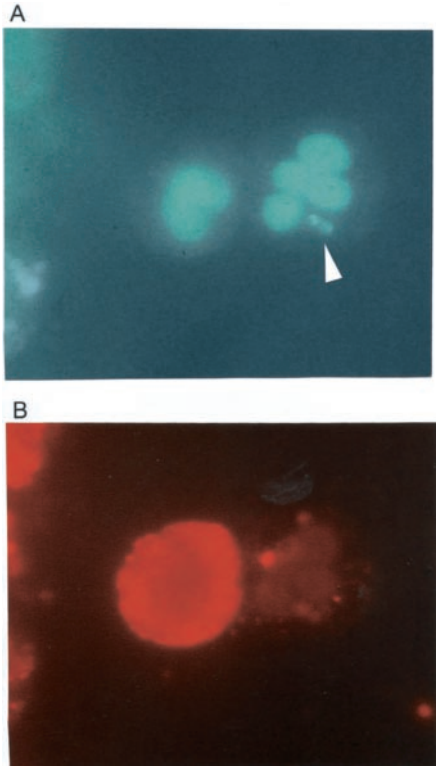

FIGURE 6. Double staining of viable and apoptotic cells. Neutrophils were coincubated with *L. major* for 18 h and stained with the live stain SYTO-16 to visualize viable cells, followed by a staining with annexin V-Alexa 568 to detect apoptotic cells. *A*, SYTO-16 staining: a green-stained viable intracellular *Leishmania* (arrow) in a PMN with segmented nucleus adjacent to a PMN with condensed nucleus. *B*, Annexin V staining of the cells shown in *A*. The PMN with the condensed nucleus is positive for annexin V (red staining), whereas the PMN containing the intracellular *L. major* is not apoptotic, as revealed by the lack of positive staining with annexin V.

is a strong inducer of delayed spontaneous apoptosis in neutrophils.

The activity of caspase-3 is markedly reduced in neutrophils after coincubation with L. major

Caspase-3 is one of the key enzymes involved in spontaneous apoptosis of neutrophils (34). The enzymatic activity of caspase-3 in lysates of neutrophils was measured by using a synthetic substrate of caspase-3, giving rise of fluorescent product after cleavage by the enzyme. A strong caspase-3 enzymatic activity was observed in PMN after incubation in vitro for 18 h (Fig. 7). Coincubation with *L. major* reduced the caspase-3 activity by ~50% (Fig. 7).

When the in vitro culture was extended to 42 h, a marked decrease in caspase-3 enzyme activity was observed in neutrophils incubated in the absence of *L. major* (Fig. 7). The low caspase-3 activity at this time point suggests that the cell death program had been completed in many neutrophils by 42 h of in vitro culture. Indeed, after 42 h, many cells were dead, as detected by staining with trypan blue (not shown), as the consequence of cellular disintegration of neutrophils upon extended incubation in vitro.

A delay of cell death could be observed in the presence of *L. major* promastigotes, in which the majority of neutrophils were still trypan blue negative after 42 h of culture (not shown). Importantly, neutrophils coincubated with *L. major* expressed a higher caspase-3 activity after 42 h as compared with the enzyme activity in these cells after 18 h of coincubation (Fig. 7). The level of caspase-3 activity of PMN after 42 h of coincubation with *L. major* was comparably high as the enzyme activity measured in the absence of *Leishmania* already 18 h after in vitro culture. This finding clearly shows that the apoptotic process of neutrophils is not blocked, but only delayed in the presence of *L. major*.

L. major *induces the release of IL-8, but not of GM-CSF by PMN*

Various cytokines are among the proteins that are synthesized by neutrophils. Among these, IL-8 (15) and GM-CSF (14) have been reported to be able to inhibit neutrophil apoptosis. To investigate whether *L. major* can affect PMN apoptosis by inducing the secretion of antiapoptotic cytokines, IL-8 and GM-CSF were measured in culture supernatants of neutrophils after coculture with *L. major*. Viable promastigotes induced the production of high levels of IL-8 within the first 24 h of coincubation (Fig. 8). The finding that the IL-8 content of the supernatants markedly increased between 18 and 24 h (Fig. 8) indicates clearly that the PMN were not apoptotic, but functionally still fully active during this period of coincubation. In contrast to viable promastigotes that induced IL-8 in the range of 2000–3000 pg/ml (Fig. 8), killed parasites induced only marginal (50–100 pg/ml) amounts of IL-8 in PMN cultures

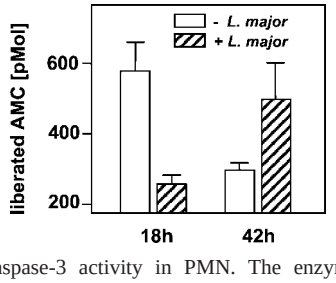

FIGURE 7. Caspase-3 activity in PMN. The enzymatic activity of caspase-3 was measured in lysates of PMN cultured in vitro for 18 and 42 h in culture medium alone or in the presence of *L. major* promastigotes at a PMN-*L. major* ratio of 1:5. The *y*-axis shows the amount of fluorescent AMC liberated from the caspase-3-specific substrate DEVD-AMC. The data shown are representative for three experiments performed.

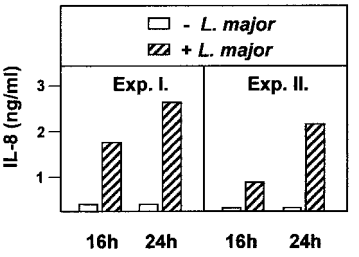

FIGURE 8. IL-8 release by PMN. Neutrophils were incubated for 18 and 24 h in culture medium alone or in the presence of viable *L. major* promastigotes at a granulocyte-*L. major* ratio of 1:5. The IL-8 content of culture supernatants was measured by ELISA.

(not shown). The GM-CSF content of all supernatants tested remained below the detection level of 10 pg/ml (not shown).

Supernatants of PMN-*L. major* cocultures were collected 24 h after coincubation and tested for antiapoptotic activity. The supernatants had no antiapoptotic effect on freshly isolated neutrophils (Fig. 2). This finding suggests that, although PMN produce IL-8 upon coculture with viable *L. major*, the induction of antiapoptotic cytokines appears not to be the mechanism of antiapoptotic effect of *Leishmania*.

Leishmania *survive in PMN in vivo*

The finding that *Leishmania* induces the delay of neutrophil apoptosis in vitro suggests that this may occur also in vivo, resulting in the survival of the parasites intracellularly in neutrophils in the first days of infection. To test this hypothesis, *L. major* promastigotes were injected s.c. into air pouches of BALB/c mice. After inflammatory stimuli, neutrophils migrate into this type of pouch within hours. This early influx of neutrophils is then followed by a massive infiltration of mononuclear cells. To investigate the intracellular survival of *Leishmania* in this in vivo model, inflammatory exudate cells were collected from the air pouch 3 days after *Leishmania* infection. Giemsa-stained cytospin slides were assessed for the presence of neutrophils. Both apoptotic and nonapoptotic neutrophil granulocytes were detected among the infiltrating cells. However, the rate of apoptotic PMN was as low as 4.6% ± 3.3%. The rate of *L. major* infection in the exudate PMN was 14.3 ± 6%. In control experiments, inert latex beads or the sterile inflammatory agent glycogen were injected into air pouches of BALB/c mice. The latex bead-induced exudate contained 10.6 ± 4.1% apoptotic neutrophils. This figure, however, is valid only for the PMN containing few beads. Because latex beads are not transparent under light microscopy, a clear differentiation between apoptotic and nonapoptotic nuclear morphology was difficult in PMN harboring several beads. In glycogen-induced exudate, the percentage of apoptotic PMN was 8.0 ± 3.4%, thus somewhat higher than in the *L. major*-induced exudate. These data suggest that *Leishmania* induce a delayed PMN apoptosis also in vivo. Importantly, intracellular parasites were seen only in nonapoptotic neutrophils, but not in those with apoptotic nuclear morphology (Fig. 9). This finding suggests that while noninfected PMN become apoptotic, the spontaneous apoptosis of infected neutrophils is delayed in vivo. It is likely that the infected nonapoptotic PMN 3 days after infection represent the PMN that ingested *Leishmania* in the first hours after infection and whose apoptosis was delayed by the parasites. The intracellular parasites were morphologically intact, demonstrating clearly that *Leishmania* can survive in neutrophil granulocytes in vivo for as long as 3 days.

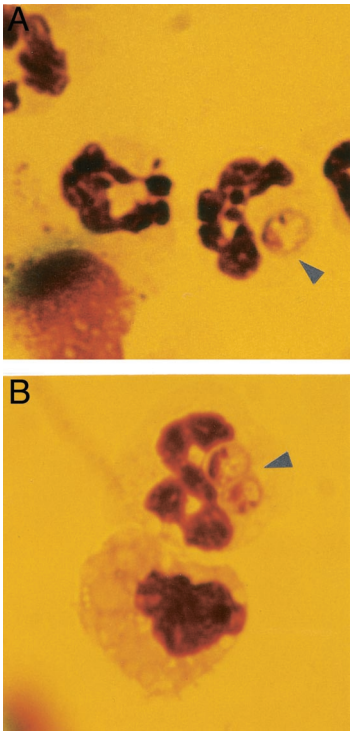

FIGURE 9. Intracellular survival of *L. major* in PMN in vivo. *L. major* promastigotes were injected into s.c. air pouch of BALB/c mice. Infiltrate cells were aspirated 3 days after infection from the air pouch. Cytocentrifuge preparates were fixed with methanol and stained with Giemsa. The arrows show nonapoptotic neutrophils with intracellular parasites.

Discussion

In this study, we report the inhibitory effect of *L. major* on the spontaneous apoptosis of neutrophil granulocytes (PMN). Previous studies from our and other laboratories revealed that PMN are the first leukocytes that appear at the s.c. site of *L. major* infection (3, 4). Recently, we demonstrated that *L. major*, after being phagocytosed by PMN, can survive in these cells intracellularly (6). Because PMN are known to have a very short $t_{1/2}$, we tested the hypothesis that *L. major* can extend the life span of PMN granulocytes.

Coincubation of PMN with *L. major* promastigotes in vitro led to an inhibition of their spontaneous apoptosis, as assessed by morphological analysis of PMN, annexin V binding, as well as by detecting the apoptosis-associated DNA fragmentation. Several intracellular microorganisms have been shown to delay the apoptosis of their host cells. *Theileria parva* infects and transforms bovine T cells. The parasite-induced mechanisms play a crucial role in the survival of transformed T cells by conveying protection against an apoptotic signal that accompanies parasite-mediated transformation (23). The molecular mechanisms underlying the inhibition of induced host cell apoptosis are not known yet. *Toxoplasma gondii* protects murine lymphoma cell lines from apoptosis, which requires the presence of live organisms (24). However, *T. gondii* inhibits the induced and not the spontaneous apoptosis of host cells. In this study, we described the inhibition of spontaneous rather than induced apoptosis of PMN by *Leishmania*. The differences between the spontaneous and induced apoptotic processes suggest that the various infectious agents may use more than one mechanism to achieve the extended survival of their host cells. Whether similar mechanisms are involved in the antiapoptotic effect of *T. gondii* and *L. major* remains to be clarified.

In our present study, we demonstrated that *Leishmania* affects the survival of neutrophils via a mechanism involving the inhibition of caspase-3 activation. Caspase-3 activation was found to be associated with the neutrophil apoptosis induced by various agents such as TNF-α treatment (35), glutathione depletion (36), or infection with *C. albicans* (21), therefore being one of the key molecules in the induced neutrophil apoptosis. In addition, the spontaneous apoptosis of PMN was also shown to involve caspase-3 (35). Freshly isolated neutrophils have a high expression of procaspase-3, which is cleaved during spontaneous apoptosis (37). We showed that the *Leishmania*-mediated delay of neutrophil apoptosis is associated with a marked decrease in caspase-3 activity. This suggests that *L. major* affects the transition of procaspase-3 to enzymatically active caspase-3. Infection with *C. albicans* was reported to enhance caspase-3 activity in PMN, resulting in the accelerated apoptosis of these cells (21). Therefore, the transition of procaspase-3 to caspase-3 appears to be a target pathway of pathogens both to inhibit and to accelerate the spontaneous apoptosis of PMN.

Previously, *Leishmania donovani* has been reported to inhibit the apoptosis of macrophages (38). However, *Leishmania* appear to use distinct pathways to inhibit apoptosis in neutrophils and macrophages. First, *Leishmania* infection induces TNF-α secretion by macrophages, and TNF-α in turn can inhibit macrophage apoptosis. Therefore, TNF-α was suggested as one possible mediator of delayed macrophage apoptosis by *L. donovani* (38). In contrast to macrophages, however, TNF-α accelerates PMN apoptosis, leading to rapid apoptotic death of these cells (35, 39). Second, *L. donovani* promastigotes were shown to induce the secretion of GM-CSF in macrophages (38), a cytokine that can delay induced apoptosis of macrophages. In our present study, PMN did not produce GM-CSF upon coculture with *L. major*. This is in accordance with previous findings that macrophages, but not neutrophils, are a major source of GM-CSF (40). Third, in our experiments, only live *L. major* promastigotes inhibited the PMN apoptosis. Killed promastigotes or a lysate of the parasites had no antiapoptotic effect. The antiapoptotic effect of *L. donovani* in macrophages did not depend on live parasites; killed promastigotes, parasite lysate, supernatants of parasite cultures as well as lipophosphoglycan had all antiapoptotic activity (38).

To date, the agent of HGE was the only known microorganism able to inhibit spontaneous neutrophil apoptosis (25). The mechanism of how this intracellular bacterium can prolong the life span of neutrophils remains fully unknown. However, it appears to be different from the mechanism used by *Leishmania*. Although only viable *L. major* displayed an antiapoptotic effect, not only live, but also killed HGE agent as well as bacterial supernatant were effective. The antiapoptotic principle of HGE agent was not identified; preformed proteins of the bacteria were suggested to be responsible for the observed effect (25). The reported data did not rule out that LPS or related bacterial products, such as the major surface protein P44 (41) or an atypical bacterial component found in HGE agents (42), mediate the antiapoptotic effect of these intracellular bacteria. This could explain that not only live, but killed bacteria and bacterial supernatant delayed the PMN apoptosis.

IL-8 has also been shown to delay the apoptosis of neutrophils (15, 43). Although PMN produced high amounts of IL-8 upon coculture with *Leishmania*, the IL-8-containing supernatants had no antiapoptotic effect on freshly isolated neutrophils. Therefore, the antiapoptotic effect of *Leishmania* is not mediated by soluble mediators such as IL-8. However, the issue of IL-8-mediated inhibition of PMN apoptosis is controversial. Whereas in one report (43) IL-8 was shown to inhibit PMN apoptosis in all concentrations in the range of 1–1000 ng/ml, others demonstrated that only

high, but not low concentrations (below 80 ng/ml) of IL-8 have this activity (15). The supernatants of PMN after conincubation with viable *L. major* promastigotes contained 2–3 ng/ml IL-8. In our studies, this concentration of rIL-8 did not affect PMN apoptosis, although a significant inhibition of PMN apoptosis was achieved by IL-8 concentrations higher than 100 ng/ml (not shown). It is also possible that the supernatants contain additional factors, such as TNF-α, which could promote cell death and counteract the effects of IL-8. However, using ELISA, TNF-α was not detectable in the supernatants (not shown).

After having established the inhibition of PMN apoptosis in vitro, we aimed to evaluate its relevance in the course of in vivo infection. An experimental murine model of *L. major* infection was applied to investigate whether *Leishmania* infection can delay the PMN apoptosis in vivo. Within the first 10–24 h after infection with *L. major*, PMN migrate rapidly into the infected skin. However, during the second and third day of infection, primarily macrophages are recruited and dominate in the cellular infiltrate (3). Using the s.c. air-pouch technique, infiltrate cells were collected from the infected skin 3 days after infection with *L. major*. Importantly, morphologically intact nonapoptotic PMN were observed, and a population of these cells contained intracellular *Leishmania*. The facts that, on the one hand, no apoptotic cells were among the infected ones and, in contrast, no *Leishmania* were seen in apoptotic PMN, suggest that the infection led to the extended survival of these cells in vivo. These are the first experimental data suggesting that the observed inhibition takes place not only in vitro, but also in vivo in the early phase of infection.

L. major did not block, but rather delayed the spontaneous PMN apoptosis by ~24 h. The infected cells became apoptotic during the second day of culture, as demonstrated by the high caspase-3 activity in PMN after 42 h of coincubation with *Leishmania*. Considering the different kinetics of recruitment of neutrophils and macrophages to the infected skin, the delay of PMN apoptosis can have significant consequences for disease development. No significant numbers of macrophages, the ultimate host cells of the parasites, are present in the infected skin at this early time point of infection (3, 4). It is conceivable that, in the absence of macrophages, PMN phagocytose *Leishmania* and hence provide them an intracellular niche for survival within the first hours of infection. Due to the short lifetime of PMN, however, the parasites can survive the first day intracellularly only if the PMN apoptosis is delayed. In this study, we have demonstrated that infected PMN become eventually also apoptotic, with a delay of ~24 h. PS is exposed on the surface of apoptotic PMN (29), leading to their recognition and phagocytosis by macrophages (11). Accordingly, we hypothesize that infected PMN, which become apoptotic with a delay of at least 24 h, are phagocytosed by macrophages that are recruited into the infected skin 1–2 days after infection. In this context, it is important to note that the uptake of apoptotic cells does not activate the antimicrobial effector mechanisms of macrophages (44). According to this hypothesis, the uptake of infected apoptotic neutrophils could be a way of silent entry of the parasites into macrophages. This fits into the "safe target" theory, which suggested that myeloid cells provide intracellular niche for the survival of *Leishmania* (45), and can explain the reported disease-promoting effect of neutrophil granulocytes in experimental *L. major* infection (46).

Acknowledgments

We thank Dr. Antje Müller for kindly performing the TNF-α ELISA. We also thank Nicole Jahnke and Nina Hermann for expert technical assistance.

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