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Role of Inorganic Nitrogen Oxides and Tumor Necrosis Factor Alpha in Killing *Leishmania donovani* Amastigotes in Gamma Interferon-Lipopolysaccharide-Activated Macrophages from *Lsh*^{s} and *Lsh*^{r} Congenic Mouse Strains

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The capacity of mature bone-marrow-derived macrophages and resident peritoneal macrophages from *Lsh*^{r} versus *Lsh*^{s} congenic mice to kill intracellular *Leishmania donovani* amastigotes when activated by recombinant gamma interferon-lipopolysaccharide (rIFN-γ–LPS) was examined. IFN-γ alone in doses up to 100 U/ml was unable to activate macrophages to kill *L. donovani* amastigotes in vitro; LPS was a necessary secondary stimulus. Similarly, LPS alone in doses up to 100 ng/ml produced no leishmanicidal activity. In bone marrow macrophages, a dose-dependent increase in leishmanicidal activity was observed as increasing rIFN-γ–LPS dose combinations were introduced, with *Lsh*^{r} macrophages maintaining a significant but not dramatic advantage within any particular dose combination. For peritoneal macrophages, the reverse was true, with macrophages from *Lsh*^{s} mice being more efficient at killing for doses of LPS up to 10 ng/ml with doses of rIFN-γ in the range of 11 to 33 U/ml. The degree of killing in both bone marrow and peritoneal macrophages correlated well with the levels of nitrites measured in the supernatants at 72 h, and a highly significant correlation was observed between 4-, 24-, or 72-h tumor necrosis factor alpha (TNF-α) release and nitrite production measured at 72 h. Inclusion of 200 μM N^G-monomethyl-L-arginine, a competitive inhibitor of the L-arginine-dependent pathway for the synthesis of inorganic nitrogen oxides, inhibited the killing, as did the addition of neutralizing anti-TNF-α antibody. These results are consistent with previous data showing an important autocrine role for TNF-α in enhancing production of inorganic nitrogen oxides by primed or activated macrophages. In addition, our results suggest that production of TNF-α and nitrites after priming or activation signals may be under a different regulatory control in mature bone marrow macrophages than in the resident peritoneal macrophage population.

The murine macrophage resistance gene, *Lsh/Ity/Bcg*, is of prime importance in determining the outcome of infection with a broad range of phylogenetically distinct intracellular pathogens, including *Leishmania donovani*, *Salmonella typhimurium*, *Mycobacterium bovis*, *Mycobacterium lepraemurium*, and *Mycobacterium intracellulare* (5). The precise mechanism by which expression of this resistance gene inhibits the intracellular multiplication of these pathogens is not known, although it is well established that expression of differential antimicrobial activity in macrophages from congenic *Lsh*^{r} and *Lsh*^{s} mice depends upon the type and maturity of the macrophage population being examined (14, 36, 48). This, in turn, may reflect differences in the ability of these macrophage subpopulations to respond to priming or activation signals. That *Lsh* regulates some key point in the macrophage activation pathway is clearly indicated by the quantitative differences observed for a variety of pleiotropic markers of priming or activation after *M. bovis* BCG or gamma interferon-lipopolysaccharide (IFN-γ–LPS) treatment of macrophages from the congenic mouse strains (7, 11). Working with the *L. donovani* model system, for example, Blackwell and coworkers have found that (i) LPS treatment before *L. donovani* infection in vivo overcomes the 2- to 3-day time delay usually observed before multiplication of the liver parasite population comes under *Lsh* gene control (13), (ii) IFN-γ treatment of resident peritoneal macrophages in vitro promotes differential phorbol myristate acetate-elicited respiratory burst (RB) activity (8) and up-regulation of major histocompatibility class II molecules (29), and (iii) LPS and IFN-γ together cause enhanced tumor necrosis factor (TNF) production in vitro by mature bone-marrow-derived macrophages from *Lsh*^{r} mice compared to *Lsh*^{s} congenic mice, the latter being more pronounced in macrophages preinfected with *L. donovani* amastigotes (7). Similarly, working in the context of the *M. bovis* infection model, Skamene and coworkers have reported quantitative differences in RB (15, 16), in expression of the AcM.1 (52) marker of macrophage activation (11), in down-regulation of 5′ nucleotidase (11), and in up-regulation of major histocompatibility class II molecules (46) after BCG activation in vivo or in vitro. In resident peritoneal macrophages, no quantitative differences in RB elicited by uptake of *S. typhimurium* (9) or *L. donovani* (6) by *Lsh*^{r} versus *Lsh*^{s} macrophages have been observed, and no one has yet provided evidence that reactive oxygen intermediates are involved in antimicrobial activity of *Lsh* gene-activated macrophages. Indeed, the microbistatic effect of *Lsh*^{r} macrophages against *L. donovani* is most pronounced in Kupffer cells (13, 14) which are oxidatively inactive in response to *Leishmania* spp. (6, 31) and other stimuli (18), and Denis et al. (15) have demonstrated that addition of inhibitors of the

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RB or scavengers of reactive oxygen intermediates did not alter the antimycobacterial activity of activated *Lsh*^{r} macrophages. The enhanced RB observed after priming or activation of *Lsh*^{r} peritoneal and splenic macrophages appears, therefore, to be a good marker of the change in physiological status of the macrophage but is not involved in the final effector pathway of antimicrobial activity. What then are the alternative antimicrobial pathways?

In the recent literature, intense interest has focused on the L-arginine-dependent production of inorganic nitrogen oxides (INO) as a potent antimicrobial pathway in immunostimulated macrophages (32, 49, 50). In this system, L-arginine is oxidized by an inducible NADPH-dependent enzyme (NO synthase) to yield L-citrulline, nitrite, and nitrate, with highly reactive nitric oxide (NO) produced as an intermediate (27, 37). NO mediates L-arginine-dependent tumor cytostasis during coculture with IFN-γ and LPS-activated macrophages, possibly via nitrosylation reactions which remove labile iron atoms from Fe-S prosthetic groups of aconitase and complexes I and II of the mitochondrial electron transport system (21, 22, 51). INO have also been implicated in the microbistatic effect of activated macrophages against the fungal pathogen *Cryptococcus neoformans* (23). More recently, INO have been demonstrated to play a role in the cytostatic or cytocidal activities of macrophages against the intracellular pathogens *Toxoplasma gondii* (1) and *Leishmania major* (25, 33, 35). The latter observation has not yet been extended to *L. donovani*. In the context of the *L. major* model, Liew et al. (33, 34) have shown that, in the presence of 10 ng of LPS per ml, recombinant TNF-α (rTNF-α) acts synergistically with IFN-γ to give maximal production of INO and antileishmanial activity. An autocrine role for TNF-α was proposed by Green et al. (24), who showed that neutralizing anti-TNF-α antibodies blocked production of INO and antileishmanial activity in IFN-γ-primed peritoneal macrophages. In this case, the parasite itself acted as the stimulus for TNF-α release by the macrophage. Many previous studies (28, 30, 40, 41, 43) have stressed the role of IFN-γ in activating macrophages for leishmanicidal activity in vitro, but fewer have examined in detail the specific dependence on LPS as the costimulus for this activation. Certainly it is well established that LPS acts as a potent stimulus for TNF-α release (3), but the ability of this LPS-induced TNF-α production to act in an autocrine fashion may be complicated by the observation that LPS also down-regulates TNF-α receptors on macrophages (20). In studies presented here, the *L. donovani* infection model is used to examine three aspects of *Lsh* gene-regulated antileishmanial activity: (i) can macrophage populations of varying maturity from *Lsh*^{r} and *Lsh*^{s} mice be activated for differential leishmanicidal activity by using IFN-γ–LPS in vitro, (ii) to what extent is this LPS-dependent, and (iii) does this leishmanicidal activity against *L. donovani* involve the LPS-dependent TNF-α and INO antimicrobial pathways?

MATERIALS AND METHODS

Mice. C57BL/10ScSn [B10(*Lsh*^{s})] mice were purchased at 4 to 6 weeks of age from Harlan Olac Ltd. (Bicester, Oxon, United Kingdom) and housed under conventional animal house conditions at the London School of Hygiene and Tropical Medicine (LSHTM) for a minimum of 4 weeks before use or bred at the LSHTM from stock originally obtained from Olac. N10 congenic B10.L-*Lsh*^{r} mice, produced as described previously (8), were bred at the LSHTM. Mice were matched by age (±2 weeks) and sex within each experiment as indicated.

Media and reagents. RPMI 1640 (Dutch Modification, ICN Flow Laboratories, Irvine, Scotland), with 20 mM L-glutamine, 10 mM sodium pyruvate, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (referred to hereafter as RPMI) was used and supplemented further with fetal calf serum (FCS, low endotoxin; Sigma Chemical Co., Poole, Dorset, United Kingdom) and D-glucose (45% solution; Sigma) as indicated. L-NMMA and D-NMMA were kindly provided by H. Hodson (Wellcome Research Laboratories, Beckenham, Kent, United Kingdom). rIFN-γ and TNF-α were obtained through Gunter Adolf of Boehringer Ingelheim (Ingelheim, Germany). Monoclonal anti-mouse TNF-α (TN3) (47) was kindly provided by Robert Shreiber (Department of Pathology, Washington University Medical School, St. Louis, Mo.). Rabbit anti-mouse TNF-α for the enzyme-linked immunosorbent assay (ELISA) was produced at the LSHTM. Neutralizing rabbit anti-mouse TNF-α used in vitro was kindly provided by John Tite (Wellcome Research Laboratories). LPS (*Escherichia coli* O111:B4 phenolic extract, tissue culture grade, L4391; or *S. typhimurium* phenolic extract, tissue culture grade, L6143) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma.

Parasites. *L. donovani* (LV9) was maintained by bimonthly passage in Syrian hamsters. Amastigotes used for in vitro infection of macrophages were purified from hamster spleen by using a modification of the method previously described (12). Briefly, the hamster spleen was homogenized in RPMI 1640–10% FCS and centrifuged at 100 × *g* for 10 min to remove erythrocytes and large cell debris. Residual erythrocytes in the supernatant were lysed by the addition of 0.05% (wt/vol) saponin for 5 min. Parasites were washed by centrifugation at 2,000 × *g* for 10 min, and the saponin treatment was repeated if erythrocytes remained obvious in the pellet. Two more spins (2,000 × *g*) and washes were performed to remove saponin, and the final suspension of amastigotes was passed three times under pressure through a 26-gauge needle to disperse clumps of parasites. Parasites were counted by use of a Helber bacteriological counting chamber with Thoma ruling (Fisons Gallenkamp Express, Loughborough, United Kingdom).

Resident peritoneal macrophages. Resident peritoneal macrophages were obtained by peritoneal lavage with 5 ml of RPMI 1640–2% FCS and 10 U of heparin per ml. Over the time of the experiments reported here, the peritoneal exudate contained 49% ± 7% B10(*Lsh*^{s}) mouse macrophages and 50% ± 8% B10.L-*Lsh*^{r} mouse macrophages as determined by FACSCAN (Becton Dickinson, Oxford, United Kingdom) analysis by using the macrophage-specific F4/80 monoclonal antibody (2) and gating for forward and side scatter. Class II (monoclonal antibody M5/114-I-A^{b,d,q} I-E^{k,d}) (4) staining by FACSCAN showed a baseline of 25% ± 4% and 39% ± 5% for *Lsh*^{s} and *Lsh*^{r} macrophages, respectively, at extraction. Cells were spun once (260 × *g*, 12 min) and resuspended in RPMI 1640–10% FCS supplemented with a solution of 45% D-glucose (380 μl/100 ml) to give a final concentration of 20 mM. Cells were counted, transferred to new polypropylene tubes (Falcon 2070; Becton Dickinson), and prewarmed to 37°C for 15 min before infection with *L. donovani* amastigotes (also prewarmed to 37°C). Amastigotes and peritoneal exudate cells were mixed in a ratio of 2:1 and left standing for 30 min at 37°C, with mixing by inversion of the tubes at 15 min. After infection, cells were spun (260 × *g*, 10 min) at room temperature, and

the supernatant containing free amastigotes was aspirated. The cell pellet was initially gently resuspended in 1 ml of fresh RPMI 1640–10% FCS plus supplements, and the volume was made up to 10 ml before spinning (260 × *g*, 10 min) was performed. The procedure was repeated twice to ensure that all free amastigotes were removed. The final pellet was gently resuspended first in 1 ml and then made up to 5 ml and recounted. The concentration of cells was adjusted to between 1.5×10^6 and 2×10^6 (constant within each experiment) in 150 μl, which was added as a "bubble" to 13-mm-diameter Thermolux coverslips (Miles Scientific) in 24-well tissue culture plates (Nunc (distributor); GIBCO, Paisley, United Kingdom). Bubbles were left for 45 min, after which the wells were flooded with 0.5 ml of supplemented RPMI 1640–10% FCS and left standing for an additional 1 h at 37°C in 5% CO_2. Wells were then washed three times with RPMI 1640 before the addition of 0.5 ml of supplemented RPMI 1640–10% FCS to each well. rIFN-γ (final concentration, 2×) with or without D- or L-NMMA (final concentration, 2×) and/or normal rabbit serum (NRS) or neutralizing rabbit anti-mouse TNF-α (final concentration, 20 μg/ml; kindly provided by J. Tite, Wellcome Research Laboratories) was added to 0.5 ml of supplemented RPMI 1640–10% FCS, and then 10 μl of LPS (final concentration, 10×) was added. Cultures were incubated for 72 h at 37°C in 5% CO_2. Supernatants were collected for TNF-α or nitrite assays at 4, 24, or 72 h as indicated. At 72 h, coverslips were washed, methanol fixed, and stained with Giemsa for enumeration of parasites.

Bone-marrow-derived macrophages. In initial experiments, mature bone-marrow-derived macrophages were cultured and infected, and leishmanicidal activity was determined by using a colorimetric (MTT) assay to determine metabolically active parasites after macrophage lysis and culture of promastigotes as described previously (30). As with peritoneal assays, bone marrow macrophages were infected in suspension (1:1, amastigotes to macrophages) and plated in 96-well plates before the addition of priming (IFN-γ) and activating (LPS) signals over the same dose range used in peritoneal cell experiments. In other experiments, bone marrow macrophages were plated onto coverslips (2×10^5 per well) in 24-well plates and parasite survival or killing was assessed on Giemsa-stained preparations as described for peritoneal cells.

TNF-α ELISA. TNF-α in culture supernatants was measured by capture ELISA by using the monoclonal antibody TN3 (10 μg/ml) to coat 96-well plates (Nunc-immunoplate maxisorb; Nunc) and the rabbit anti-TNF-α polyclonal antibody (1:200) for second-layer detection. Goat anti-rabbit immunoglobulin G (H and L chains) peroxidase conjugate was used, and the substrate was 2,2′-azino-di-[3-ethyl-benzthiazoline sulfonate] (ABTS) with hydrogen peroxide (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.). Supernatants were titrated from undiluted to 1:3, 1:9, and 1:27. TNF-α units per milliliter were calculated from a standard curve (2.5 to 50 U/ml) of rTNF-α run on each plate.

Measurement of nitrites. Griess reagent (1% sulfanilamide [Sigma], 0.1% naphthylethylenediamine hydrochloride [Sigma], 2.5% orthophosphoric acid [Analar B.D.H., Dagenham, Essex, United Kingdom]) was freshly made prior to use. Griess reagent was added 1:1 with supernatant and left standing for 5 min at room temperature. Standards prepared by using sodium nitrite (2 to 100 μM) were included on each assay plate. A_{570} values were read on a spectrophotometer (Dynatech or Multiskan).

Estimate of cell densities after treatments. In some experiments an estimate of cell density after treatment was obtained by methanol resolubilization of nuclear Giemsa-stained wells and reading A_{540} values. Cell densities per well were calculated from a standard curve generated by titrating known numbers of peritoneal macrophages freshly adhered to 96-well plates.

RESULTS

IFN-γ–LPS dose response for leishmanicidal activity. Figure 1 shows the dose response for IFN-γ–LPS priming or activation for leishmanicidal activity for mature bone marrow macrophages from congenic *Lsh*^{r} and *Lsh*^{s} mice. By using the MTT assay, leishmanicidal activity was measured as a decrease in optical density reading, and the data are presented as a percentage of the untreated control, 0:0 IFN-γ–LPS reading. In the absence of IFN-γ, less than a 20% reduction in optical density was observed over the dose range of 0 to 100 ng of LPS per ml, indicating that LPS alone was an insufficient signal to result in significant parasite killing in macrophages from either mouse strain. Similarly, IFN-γ alone, in doses up to 100 U/ml, was unable to activate bone marrow macrophages in vitro to kill *L. donovani* amastigotes. When LPS was added as a secondary stimulus, macrophages from both mouse strains could be activated to a leishmanicidal state. At 0.1 ng/ml, only *Lsh*^{r} macrophages became partially leishmanicidal and then only at the highest dose of IFN-γ. A dose-dependent increase in leishmanicidal activity was observed in macrophages from both mouse strains for increasing concentrations of IFN-γ–LPS, with *Lsh*^{r} macrophages maintaining the advantage within any dose combination for IFN-γ–LPS. In two repeat experiments where parasite killing was determined microscopically, enhanced leishmanicidal activity was observed in *Lsh*^{r} bone marrow macrophages over those of *Lsh*^{s} over the dose range 0.1 to 10 ng of LPS per ml in combination with 33 U of IFN-γ per ml (data not shown). For peritoneal macrophages, LPS alone had variable effects on parasite survival in macrophages from the two mouse strains. Although significant leishmanicidal activity was not observed with LPS alone, high doses of LPS (≥10 ng/ml) sometimes enhanced parasite survival relative to the 0:0 IFN-γ–LPS control. Hence, the superimposing effects of IFN-γ on leishmanicidal activity could only be compared if results were expressed as a percentage of the count at 0 U of IFN-γ per ml within each dose of LPS (Fig. 2). LPS was again seen to be necessary for induction of antileishmanial activity, since IFN-γ alone in doses up to 100 U/ml had no significant effect on antileishmanial activity in either strain. With the addition of graded doses of LPS from 0.1 to 100 ng/ml, macrophages from both mouse strains became leishmanicidal. In this case, however, it was macrophages from *Lsh*^{s} mice which appeared to be more efficient at killing than *Lsh*^{r} macrophages for doses of LPS up to 10 ng/ml over the IFN-γ dose range of 11 to 33 U/ml. Similar data were obtained in a repeat experiment. At low doses of LPS, high-dose (100 U/ml) IFN-γ inhibited macrophage activation for leishmanicidal activity, a phenomenon we observed again in the repeat experiment here and in the context of other parameters for macrophage priming or activation previously (8, 7).

Relationship between leishmanicidal activity and nitrite production. For both peritoneal and bone marrow macrophage experiments, nitrites present in the supernatants of infected primed or activated macrophages were measured at 72 h in parallel with assessment of leishmanicidal activity. For each dose of LPS, the curve (Fig. 2) for nitrite levels

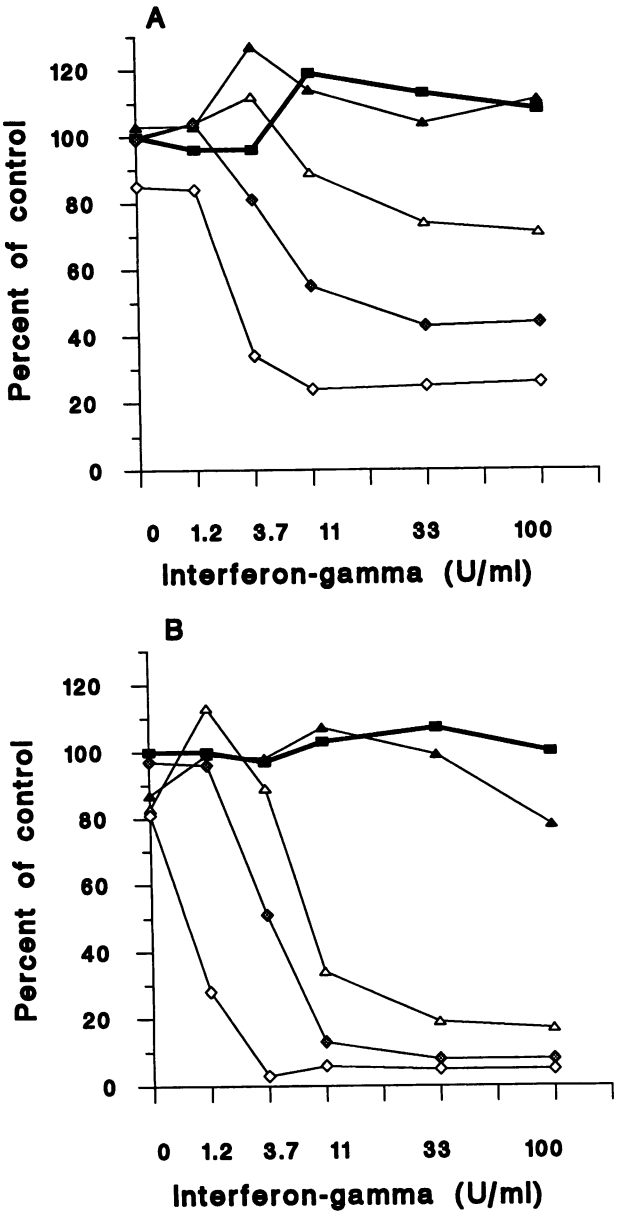

FIG. 1. Leishmanicidal activity for mature bone-marrow-derived macrophages from B10(*Lsh*^{s}) (A) and B10.L-*Lsh*^{r} (B) congenic mice. Macrophages were primed or activated with various dose combinations of IFN-γ (*x* axis) and LPS (concentrations, 0 [■], 0.1 [▲], 1 [△], 10 [◈], and 100 [◇] ng/ml), and the numbers of surviving parasites were determined at 72 h by lysis of macrophages, culture of released promastigotes, and incorporation of MTT into metabolically active parasites. Resulting optical density readings are expressed as a percentage (mean ± standard deviations [all <10% of the mean]) of the 0:0 IFN-γ–LPS reading.

followed the inverse for the IFN-γ-induced leishmanicidal curve, 100 U of IFN-γ per ml resulting in a reduction of the level of nitrites present in the supernatants at low doses of LPS. IFN-γ alone could stimulate a modest increase in nitrite production by macrophages, but there was clearly a threshold level below which no leishmanicidal activity was observed. When the data are examined for all IFN-γ–LPS dose combinations (Fig. 3), there is a significant correlation between nitrite production and degree of leishmanicidal activity for both macrophage types and both mouse strains, the relative positions of the regression lines reflecting the difference between *Lsh*^{r} and *Lsh*^{s} macrophages according to their origin as discussed above. To determine more definitively whether the INO pathway was important for leishmanicidal activity, additional experiments were performed examining leishmanicidal activity in primed or activated macrophages in the presence of inhibitors of the INO pathway.

INO mediate leishmanicidal activity in primed-activated peritoneal macrophages. Peritoneal macrophages infected with *L. donovani* amastigotes were activated in vitro with IFN-γ and LPS in the presence of the enantiomers D-NMMA and L-NMMA, the former acting as a control for the L-NMMA which acts as a competitive inhibitor of L-arginine metabolism by the enzymes of the INO pathway. In the presence of 200 μM L-NMMA, the capacity of both *Lsh*^{r} and *Lsh*^{s} macrophages to kill amastigotes when activated with increasing concentrations of IFN-γ in the presence of 1 (data not shown) or 10 ng of LPS per ml (Fig. 4) was totally inhibited, whereas 200 μM D-NMMA had no effect on leishmanicidal activity. Nitrite production was inhibited by L-NMMA for macrophages from both mouse strains over the IFN-γ–LPS dose ranges employed. Similar results were obtained in bone marrow macrophage experiments employing the inhibitor L-NMMA (Table 1), thus indicating that INO play an important role in leishmanicidal activity of both peritoneal and bone marrow macrophage populations.

Role of TNF-α in INO-mediated leishmanicidal activity. In our previously reported study of bone-marrow-derived macrophages (7), a clear dose-dependent difference in TNF-α release over 24 h was observed when macrophages from *Lsh*^{r} and *Lsh*^{s} congenic strains were activated with increasing amounts (0.1 to 1,000 ng/ml) of LPS in the presence of 25 U of rIFN-γ per ml. The magnitude of this response was enhanced in macrophages preinfected with *L. donovani* amastigotes, suggesting that the parasite itself may act as a trigger for TNF production and release. *Lsh*^{r} macrophages showed significantly higher TNF-α release throughout, which would account for the enhanced leishmanicidal activity observed in experiments reported here if TNF-α performs an essential autocrine function in production of INO. Figure 5 again confirms a direct correlation between 4-h (or 72-h; results not shown) TNF-α release and 72-h nitrite production, both for uninfected resident peritoneal macrophages and for infected bone marrow macrophages. A definitive autocrine role for TNF-α in stimulating production of INO and leishmanicidal activity is shown in Table 1; neutralizing anti-TNF-α antibody clearly blocks detection of TNF-α release, nitrite production, and leishmanicidal activity. In the same experiment, L-NMMA again inhibited nitrite production and leishmanicidal activity but had no effect on the detection of TNF-α release.

DISCUSSION

Experiments reported here demonstrate the following: (i) that resident peritoneal and mature bone-marrow-derived macrophages from *Lsh*^{r} and *Lsh*^{s} congenic mouse strains respond differentially, and in a dose-dependent manner, to activation by IFN-γ and LPS for antileishmanial activity; (ii) that activation to a leishmanicidal state is LPS dependent; and (iii) that macrophages from both *Lsh*^{r} and *Lsh*^{s} macrophages kill *L. donovani* amastigotes intracellularly via the

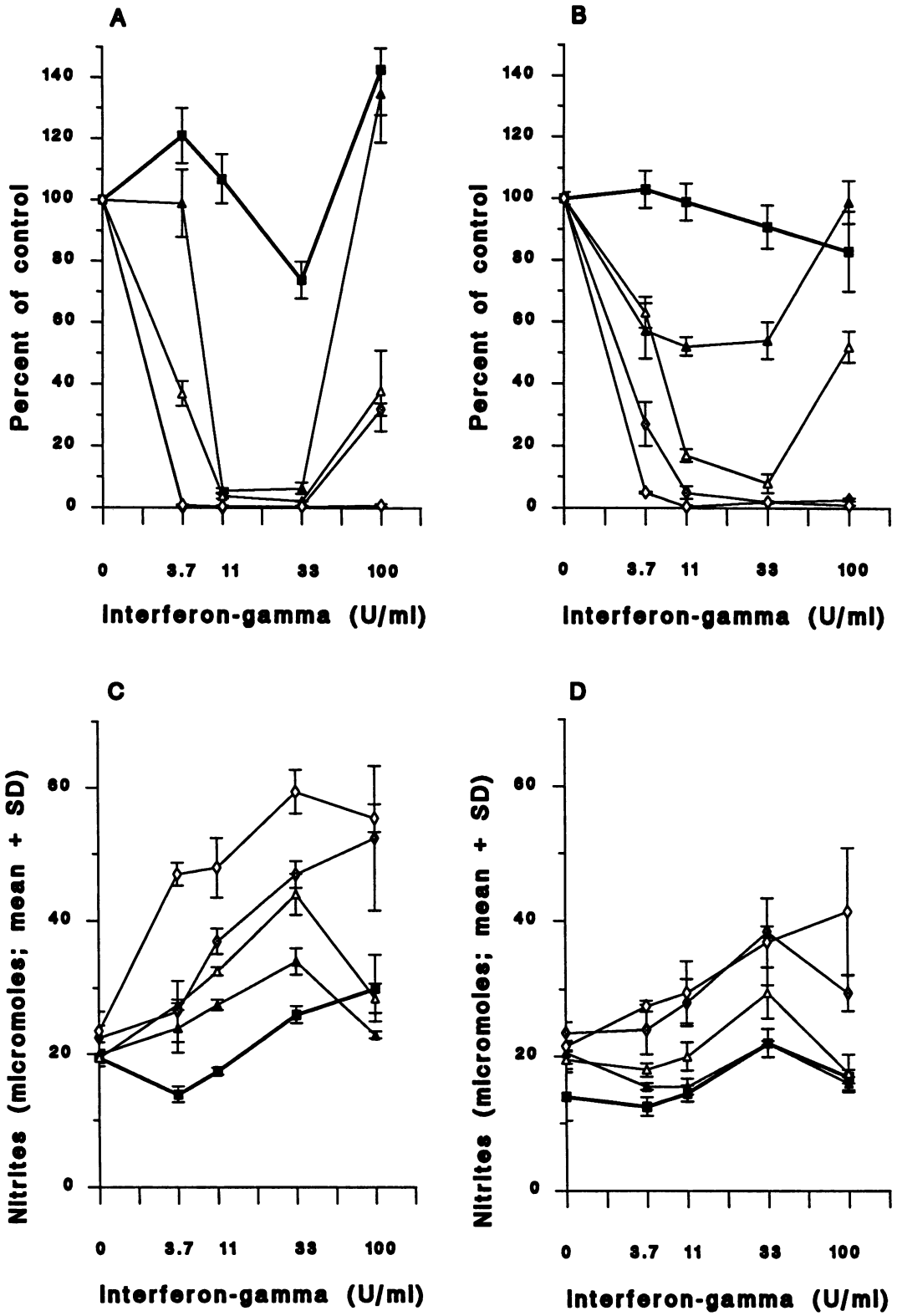

FIG. 2. Leishmanicidal activity (A and B) and associated nitrite release (C and D) for peritoneal macrophages from congenic B10(*Lsh*^{s}) (A and C) and B10.L-*Lsh*^{r} (B and D) mice primed or activated with various dose combinations of IFN-γ (*x* axis) and LPS (concentrations, 0 [■], 0.1 [▲], 1 [△], 10 [◇], and 100 [◈] ng/ml). Leishmanicidal data were based on duplicate counts (minimum of 100 macrophages scored for each count) for duplicate coverslips for each treatment. Baseline counts (amastigotes per 100 macrophages [mean ± standard error]) in untreated macrophages were 155 ± 33 for *Lsh*^{s} and 207 ± 35 for *Lsh*^{r} mice on day zero and 106 ± 11 and 205 ± 17, respectively, at 72 h. Data are presented as a percentage of the 72-h zero IFN-γ reading within each dose of LPS. Nitrites (micromoles per milliliter [mean ± standard deviation]) were measured in parallel from culture supernatants. Mice used in this experiment were 12 to 14 weeks old.

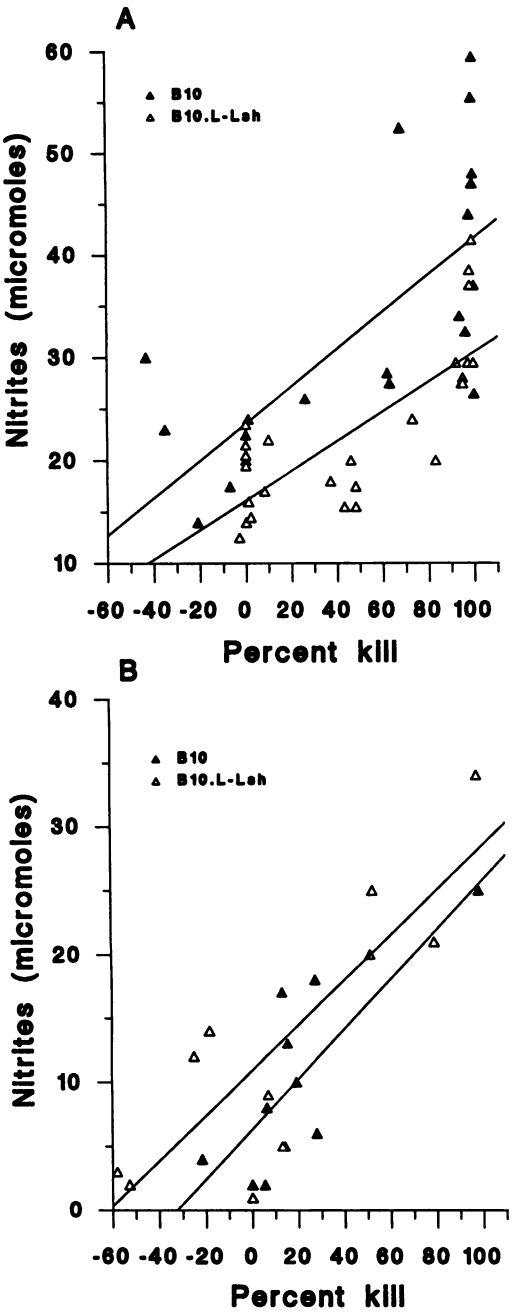

FIG. 3. Correlation between nitrite release (micromoles per milliliter) and leishmanicidal activity (percent kill) in peritoneal (A) and bone-marrow-derived macrophages (B) from Lsh^s and Lsh^r mouse strains. For peritoneal macrophages, r equals 0.5334 ($P < 0.01$, $n = 25$) and 0.5853 ($P < 0.01$, $n = 25$) for Lsh^s and Lsh^r mice, respectively. Comparable values for bone-marrow-derived macrophages were $r = 0.6061$ ($0.01 < P < 0.05$, $n = 11$) and 0.7185 ($0.01 < P < 0.05$, $n = 11$).

L-arginine-dependent production of INO inhibitable by L-NMMA. The latter observation is of particular significance, since it had always been difficult to rationalize the enhanced *Lsh* gene-mediated antileishmanial activity of the resident Kupffer cell population (13, 14, 42) with reports that this macrophage population has virtually no capacity for the production of reactive oxygen intermediates (18) known from previous studies to be a potent antileishmanial mechanism of activated macrophages (38, 39). This "defect" or down-regulation of RB in Kupffer cells is believed to be related to the major housekeeping role they play in the clearance of LPS, immune complexes, and senescent erythrocytes, which they can then perform without oxidative damage to bystander cells. When treated in vitro with recombinant IFN-γ alone, Kupffer cells are able to respond in terms of up-regulated class II molecule expression. However, they fail to make a RB response and are unable to kill *T. gondii* tachyzoites or *L. donovani* promastigotes (31). By using lymphokine supernatants from concanavalin A-stimulated spleen cells, Olivier et al. (42) were able to demonstrate differential leishmanicidal activity in Kupffer cells from Lsh^r C57L/J versus Lsh^s C57BL/6J mice. No information was given on the LPS environment during production of the spleen cell supernatant or in the Kupffer cell activation experiments themselves.

In studies presented here, we found LPS to be a necessary cosignal with IFN-γ for the activation of macrophages to kill intracellular *L. donovani* amastigotes. For peritoneal macrophages, low (0.1 to 1.0 ng/ml) doses of LPS in conjunction with high (100 U/ml) doses of IFN-γ generally depressed antileishmanial activity. This reversal of antileishmanial activity at high doses of IFN-γ was not observed with bone-marrow-derived macrophages, a factor which might be related to lack of preexposure to LPS as compared with the resident peritoneal populations extracted from mice reared under conventional animal house conditions. This prior "experience" of the peritoneal macrophages in vivo might also explain our failure here and previously (14) to demonstrate an antileishmanial advantage for Lsh^r over Lsh^s peritoneal macrophages. Indeed, the reverse appeared to be true, with peritoneal macrophages from Lsh^s mice responding more efficiently to IFN-γ–LPS priming or activation for TNF-α release, nitrite production, and antileishmanial activity. The anomaly goes further, since the class II expression data (25% ± 4% Lsh^s:39% ± 5% Lsh^r) at extraction suggests that the Lsh^r macrophages had received greater prepriming in vivo. Recent studies (18–20) have, however, focused on down-regulation of macrophage activation, and our own results here confirm that high doses of priming or activation signals can switch off the activation process. Perhaps the prior priming in vivo has made the Lsh^r peritoneal macrophage more sensitive to this down-regulation in response to further stimulation in vitro. Although consistent with our previous observation that only the mature resident tissue macrophage expresses *Lsh* gene-mediated resistance, the magnitude of the advantage observed here for mature Lsh^r bone marrow macrophages is not dramatic and seems inadequate to account for the 1.5 to 2 logs of difference in parasite loads observed 8 to 15 days after infection in vivo (10). One hypothesis currently being explored is that of differential down-regulation of responsiveness to the autocrine TNF-α loop. This could involve differential down-regulation of LPS or TNF-α receptors, or a cyclic AMP (cAMP)-dependent down-regulation of TNF-α production. In preliminary experiments (45a), we have established that either addition of exogenous prostaglandin E2 or inhibition of cAMP catabolism with theophylline reduces TNF-α release. The parasite itself may also contribute to both positive and negative regulatory pathways. Previous studies (44, 45) have shown, for example, that *L. donovani* is a potent stimulator of prostaglandin E2 synthesis in BALB/c (Lsh^s) macrophages and that preinfection of macrophages selec-

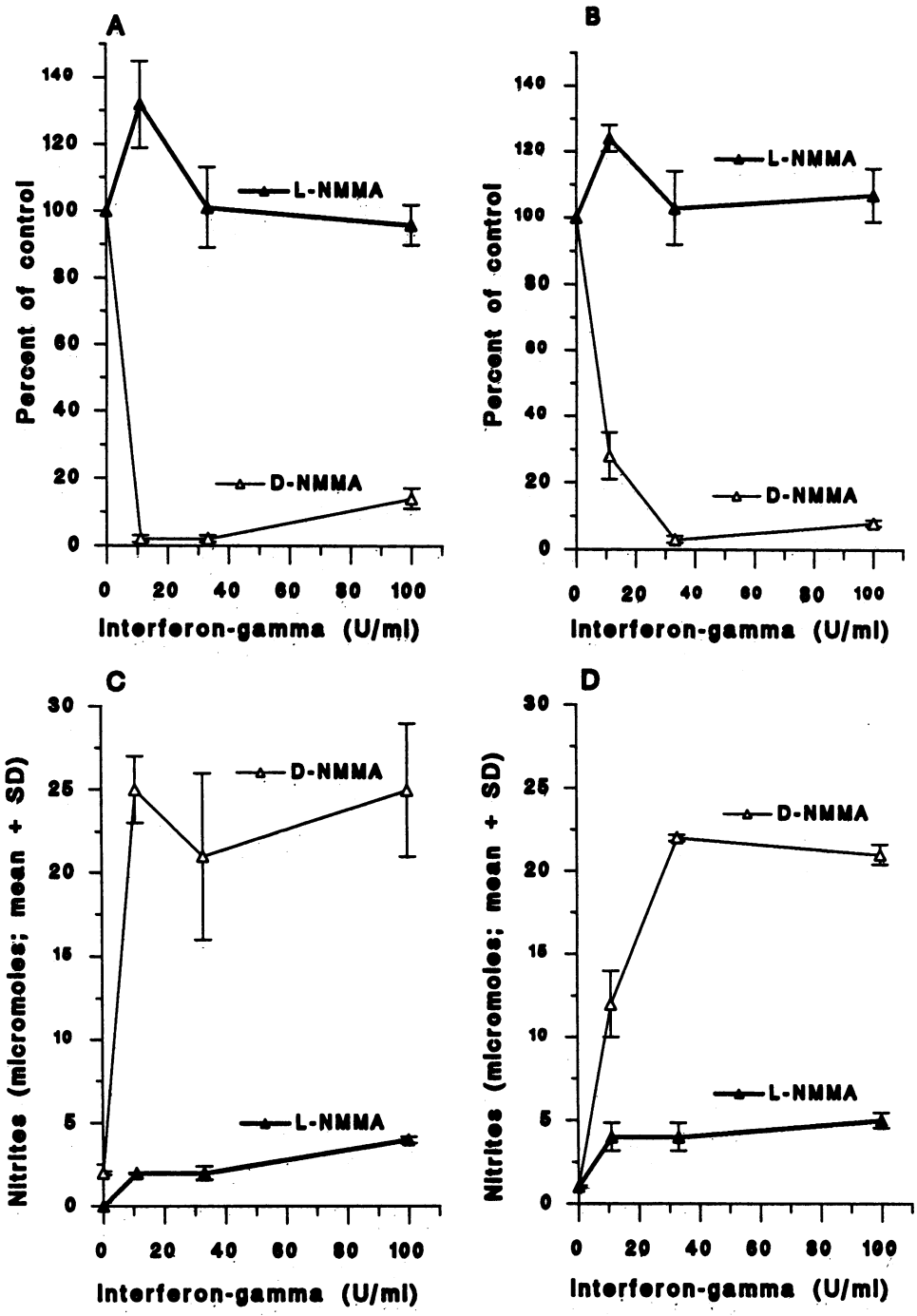

FIG. 4. Leishmanicidal activity and nitrite release for peritoneal macrophages from B10(*Lsh*^{s}) and B10.L-*Lsh*^{r} mice incubated with 200 μM D- or L-NMMA and activated with increasing doses of IFN-γ in combination with 10 ng of LPS per ml. Data were based on duplicate counts (minimum of 100 macrophages scored for each count) for duplicate coverslips for each treatment. Day zero baseline counts for parasite uptake were 208 ± 50 and 207 ± 24 amastigotes per 100 macrophages for *Lsh*^{s} and *Lsh*^{r} mice, respectively. Baseline counts at 72 h for 0 IFN-γ were 194 ± 7 (D-NMMA) and 237 ± 22 (L-NMMA) for *Lsh*^{s} mice and 326 ± 28 (D-NMMA) and 327 ± 14 (L-NMMA) for *Lsh*^{r} mice. Data are presented as a percentage of the 72-h zero IFN-γ count within each treatment. Nitrite release (micromoles per milliliter [mean ± standard deviation]) was measured in parallel from culture supernatants. Four- to 6-week-old mice were used in this experiment.

tively diminishes interleukin-1 (but not TNF-α) production. More recently, Descoteaux et al. (17) have shown that parasite-derived lipophosphoglycan stimulates rapid expression of both c-*fos* and TNF-α genes, but that preexposure to lipophosphoglycan renders the cells refractory to LPS- or protein kinase C-dependent signal transduction pathways. Lipophosphoglycan induced a rapid down-modulation of TNF-α receptors but did not impair stimulation of expression of c-*fos* by the cAMP analog, dibutiryl cAMP. Work in our laboratory is also examining regulation of expression of

TABLE 1. Effect of L-NMMA (200 μM) and neutralizing rabbit anti-mouse TNF-α on 24-h TNF-α release, 72-h nitrite production, and 72-h parasite kill in mature bone-marrow-derived macrophages from B10(*Lsh*^{s}) and B10.L-*Lsh*^{r} mice activated with 33 U of IFN-γ per ml and 1 ng of LPS

Strain	Treatment (IFN-γ:LPS)	24-h TNF-α release (U/ml)[b]	72-h nitrite production (μmol)[b]	No. of parasites per 100 macrophages[b]
B10	33:0 + NRS	10 ± 1.1	13 ± 0.4	35 ± 2
	33:1 + D-NMMA + NRS	66 ± 3.2	24 ± 8.1	0.6 ± 0.2
	33:1 + L-NMMA + NRS	66 ± 4.1 (0)	17 ± 1.0 (29)	36 ± 1 (103)
	33:1 + D-NMMA + anti-TNF	7 ± 0.5 (90)	10.5 ± 5.4 (56)	34 ± 2 (97)
	33:1 + L-NMMA + anti-TNF	8.5 ± 0.6 (87)	8 ± 1.4 (67)	39 ± 4 (110)
B10.L-Lsh^r	33:0 + NRS	9 ± 0.2	14 ± 0.4	29 ± 4
	33:1 + D-NMMA + NRS	81 ± 3.3	35 ± 1.8	0.5 ± 0.2
	33:1 + L-NMMA + NRS	77 ± 4.0 (5)	25 ± 0.8 (29)	14 ± 2 (46)
	33:1 + D-NMMA + anti-TNF	7.0 ± 0.4 (91)	20 ± 0.6 (43)	12 ± 2 (42)
	33:1 + L-NMMA + anti-TNF	6.5 ± 1.5 (92)	13 ± 0.6 (63)	31 ± 1 (107)

[a] Control 33:0 IFN-γ–LPS are shown for comparison. Similar inhibitory effects were observed with the treatments in B10.L-*Lsh*^{r} macrophages receiving a 33:0.1 dose combination, but this combination produced no leishmanicidal activity in B10(*Lsh*^{s}) macrophages. Similar data were obtained in a repeat of the full experiment.

[b] Numbers in parentheses show percent inhibition of the response relative to the 33:1 + D-NMMA + NRS baseline value.

the early response genes in congenic B10(*Lsh*^{s}) macrophages versus that in B10.L-*Lsh*^{r} macrophages. Further experiments are also required to determine whether other microorganisms which come under *Lsh* gene regulation are sensitive to INO-mediated killing and to what extent a human homolog for the gene would rely on production of INO as the final effector mechanism for antimicrobial killing. For the present, however, results presented here demonstrate clearly that, as for *L. major*, IFN-γ- TNF-α-stimulated production of INO by murine macrophages provides a potent leishmanicidal pathway against *L. donovani*.

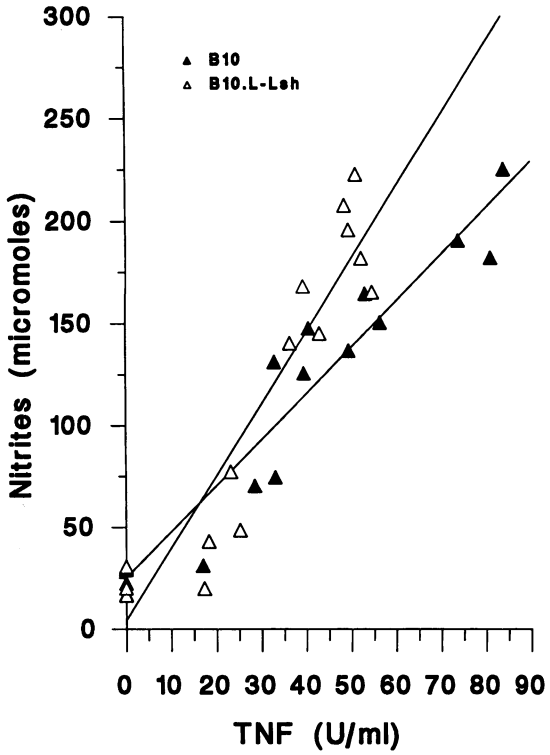

FIG. 5. Correlation between 4-h TNF-α (units per milliliter; 2 × 10^6 cells) release and 72-h nitrite (micromoles per milliliter; 2 × 10^6 cells) production for peritoneal macrophages treated with various doses of IFN-γ and LPS. r equals 0.9210 ($P < 0.01$, $n = 15$) and 0.8842 ($P < 0.01$, $n = 15$) for *Lsh*^{s} and *Lsh*^{r} macrophages, respectively. The same significance levels were obtained when 72-h TNF-α release values were compared with 72-h nitrite production values. For bone-marrow-derived macrophages (not graphed), comparable values of $r = 0.6219$ ($0.01 < P < 0.05$, $n = 10$) and 0.6311 ($0.01 < P < 0.05$, $n = 10$) were obtained. Data were corrected for the number of cells per well by using resolubilization of nuclear Giemsa staining.

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