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Lectin-Induced Nitric Oxide Production

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Considering that nitric oxide (NO) may be involved in anti-tumoral and anti-parasite lectin effects, in this report we investigated whether lectin induces NO production. Lectins from *Canavalia brasiliensis, Dioclea grandiflora, Pisum arvense* (PAA), and concanavalin A induced murine peritoneal cells to produce NO *in vitro.* PAA induced similar levels to that obtained with lipopolysaccharide plus interferon-γ. NO production by adherent cells was significantly lower than that of unfractionated cells, suggesting a combination of lectin stimuli directly on macrophages and via lymphocyte stimulation. *Ex vivo* experiments showed that cells stimulated *in vivo* could maintain NO production *in vitro* without further stimuli. NO synthesis blockage *in vivo* can significantly increase cell numbers in draining lymph nodes after lectin injection compared to unblocked controls, suggesting an *in vivo* association of lectin stimuli and NO production. Taken together these data show that lectins can induce NO production both *in vitro* and *in vivo.*

Key Words: **lectins; nitric oxide; adherent cells; peritoneal cells; macrophages; immunology; *Canavalia brasiliensis; Canavalia ensiformis;* concanavalin A; ConA; *Dioclea grandiflora; Pisum arvense.***

INTRODUCTION

Lectins form an important class of ubiquitous natural carbohydrate-binding proteins (1). Lectins such as concanavalin A (Con A), have been well-characterized and shown to possess several biological activities (2). Although the value of lectins as lymphocyte polyclonal activators is well-established (2, 3), their actions over other cells of the immune system have not been extensively evaluated.

Con Br, the lectin from *Canavalia brasiliensis*, is largely homologous to Con A, differing from it in only three residues (4). We have shown that Con Br induces a reduction in parasitism of macrophages infected with *Leishmania amazonensis.* Furthermore, when administered *in vivo* Con Br led to protection by an interferon-γ (IFN-γ)-independent mechanism (5). The direct action of lectins on macrophages, resulting in leishmania death, remains to be characterized.

Nitric oxide (NO) is an oxidizing agent, synthesized from L-arginine by different enzymes, the nitric oxide synthases (NOS). The inducible form of NOS (iNOS) is stimulated by proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), IFN-γ, or bacterial products such as lypopolysaccharide (LPS), and is inhibited by steroids (6–8). NO plays an important role in immunoregulation, controlling T-lymphocyte proliferation (9, 10), down-regulating IFN-γ (11) and IL-2 production (12), reducing leukocytes ability to act as antigen presenting cells (8), and leading to apoptosis onset (13, 14). NO is cytotoxic or cytostatic against a variety of pathogens (15, 16), including fungi, helminths, bacteria, protozoa (17), and viruses (18). It mediates host defense against *Leishmania major* (19–21), *Entamoeba histolytica* (22), *Trypanosoma cruzi* (23), *Mycobacterium avium* (24), and other pathogens. NO is also involved in cytotoxity activity against tumor cells (25, 26), and it is possible that NO is implicated in tumor lysis induced by lectins which have been shown to mediate an anti-tumoral effect (27–29).

Considering the possibilities of NO involvement in anti-parasitic and anti-tumoral effects induced by lectins we decided to investigate whether stimulation with lectin could lead to NO production by murine macrophages.

MATERIAL AND METHODS

Animals. Female BALB/c mice 8–12 weeks of age were obtained from the central animal facility of FIOCRUZ (Rio de Janeiro, Brazil) and, maintained with commercial balanced mouse ration and water *ad libitum.*

Lectins. The lectin from *Canavalia ensiformes* (concanavalin A) was purchased from Sigma (Sigma, St. Louis, MO) and the lectins from *C. brasiliensis* (Con

Br), *Dioclea grandiflora* (DGL), and *Pisum arvense* (PAA) were obtained from legume seeds according to standard techniques (30). Lectin preparations were tested to exclude LPS contamination (data not shown).

In vitro experiments. Resident peritoneal cells were obtained by peritoneal lavage with cold phosphate-buffered saline (PBS) (Sigma) and washed three times in RPMI 1640 medium (Gibco BRL, Rockville, MD). Approximately 10^5 cells were cultivated in 96-well plates with 200 μl RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Sigma), penicillin (10 U/ml) (Gibco BRL) and streptomycin (10 μg/ml) (Gibco BRL). Cells were maintained without stimulus or were stimulated with the previously mentioned lectins at concentrations of 2.5, 5, 10, 25, 50, 75, or 100 μg/ml. Positive control wells received LPS (10 ng/ml) (Sigma) plus recombinant rat IFN-γ (100 U/ml) (Institute Roussel Uclaf, Romainville, France). Rat IFN-γ, used *in vivo* in mice, stimulates the respiratory burst of murine peritoneal macrophages at an optimal dose of 5000 IU/animal (Dr. Michel Lando, personal communication). Each condition was evaluated in triplicate. Unless otherwise indicated, supernatants were tested after 48 h of culture. In some experiments nonadherent cells were removed by extensive washings with warm RPMI 1640 medium before stimulation.

Ex vivo experiments. Groups of three BALB/c mice were injected ip with 100 μg of lectin (Con A, Con Br, or PAA) diluted in PBS (5) or with PBS alone as control. A pool of peritoneal cells was obtained 6 h after administration and incubated for 48 h without further incubation with lectins.

NO production assessment. The presence of NO in supernatants was evaluated by nitrite concentrations using the Griess reaction (31, 32). Phosphoric acid, *N*-(1-naphthyl)ethylenediamine and sulfanilamide were purchased from Sigma.

NO blockage. BALB/c mice were injected twice ip with 7 μg/200 μl PBS of the selective inhibitor of iNOS aminoguanidine (Sigma) (33), 24 h before and at the time of lectin sc injection in mice left footpads and PBS injection in the right ones. Fifteen hours after lectin stimulation lymph nodes were collected as described below.

Lectin stimulation of lymph node cells. Lymph node cells were stimulated with lectins as previously described (34). Briefly, 50 μg of lectin (in 20 μl) was injected subcutaneously in the left hind footpads of BALB/c mice, and 20 μl of PBS was injected in the contralateral ones as a control. The draining lymph nodes were removed after 15 h, and the number of cells obtained in each lymph node was counted. Results are expressed as the ratio of treated (lectin or lectin + aminoguanidine) over control (PBS) lymph node cell numbers.

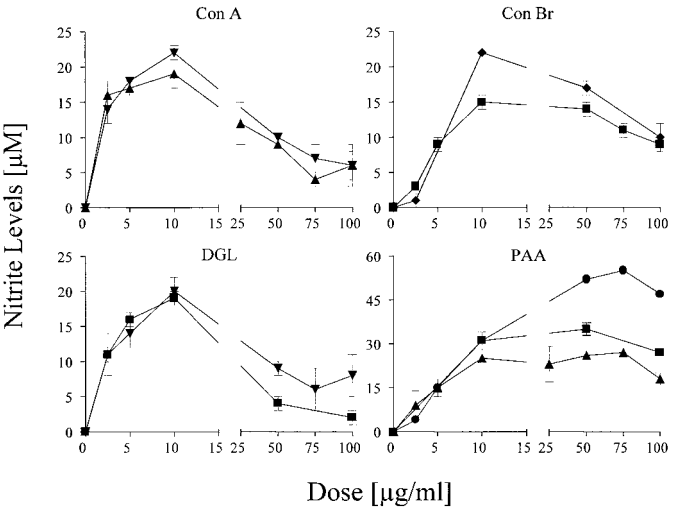

FIG. 1. Levels of nitrite in the supernatants of murine peritoneal cell cultures stimulated with different doses of legume lectins. Peritoneal cells were stimulated *in vitro* with 0, 2.5, 5, 10, 25, 50, 75, and 100 μg/ml of Con A, Con Br, PAA, or DGL. Supernatants (48 h) were harvested and nitrite levels were estimated through the Griess reaction. Each curve represent a different assay (mean of a triplicate ± SD). A positive control (IFN-γ + LPS) and a negative control (unstimulated) were included in each experiment but results are not shown.

Statistical analysis. Results were compared with Student's *t*-test, using GraphPad Prism software (version 2.00, GraphPad Software Incorporated, San Diego, CA), and differences were considered significant if $P < 0.05$.

RESULTS

In vitro lectin-induced NO production. Dose–response curves of resident peritoneal unfractionated cells stimulated with all tested lectins peaked at the concentration of 10 μg/ml, but three different patterns of response could be demonstrated (Fig. 1). A time-course evolution showed that NO is poorly detectable 24 h after lectin stimulation. Significant responses were observed at 48 h ($P < 0.0001$). At 60 h poststimulation, all lectins have induced significantly increased NO production when compared with levels obtained at 48 h ($P < 0.01$, Fig. 2). Data depicted in Fig. 2 also demonstrate the high capacity of lectin PAA in inducing NO production. Levels obtained with PAA stimulation were very similar to those obtained with LPS + IFN–γ without significant differences between them at all time points evaluated.

Direct lectin effect on adherent cells. In order to investigate a possible direct effect of lectins on macrophages, we compared NO production by unfractionated or adherent peritoneal cell populations. Adherent cells produce NO, although significantly lower amounts than unfractionated cells ($P < 0.01$, Fig. 3). This indicates that lectins

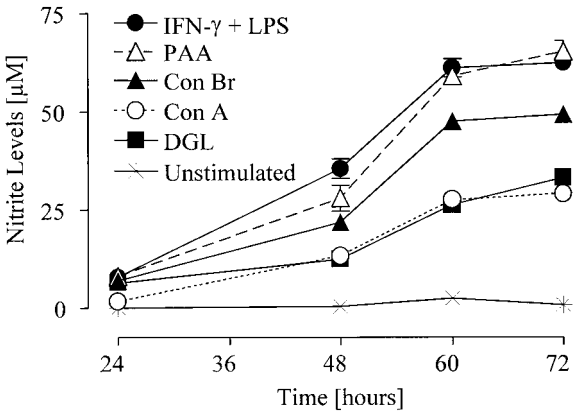

FIG. 2. Kinetics of nitrite production after stimulation of peritoneal cells by legume lectins. Peritoneal cells were stimulated *in vitro* with 10 μg/ml of lectins and cultivated for 24, 48, 60, and 72 h. The Griess reaction was performed to determine nitrite levels. Each point represents the mean of two assays in triplicate ± SD.

stimulate macrophages directly, but lectin-stimulated lymphocytes increase NO production.

In vivo administration of lectin (100 μg/animal ip) did not lead to detectable serum nitrite/nitrate levels (measured at 6, 15, and 24 h, data not shown). The absence of detectable NO end products in serum after stimulation may be a result of dilution and therefore *in vivo* NO production cannot be ruled out.

Ex vivo lectin-induced NO production (Fig. 4). To evaluate whether an *in vivo* treatment leads to cell activation we have performed *ex vivo* experiments. Lectins (100 μg) were injected ip and peritoneal cells were collected 6 h later and cultivated *in vitro* for 48 h without further stimulation. Figure 4 shows that peritoneal cells produce significant amounts of NO following *in vivo* lectin stimulation ($P < 0.0001$). In contrast to *in vitro* stimulation, Con Br-induced NO production was significantly greater than that induced by PAA ($P < 0.0001$).

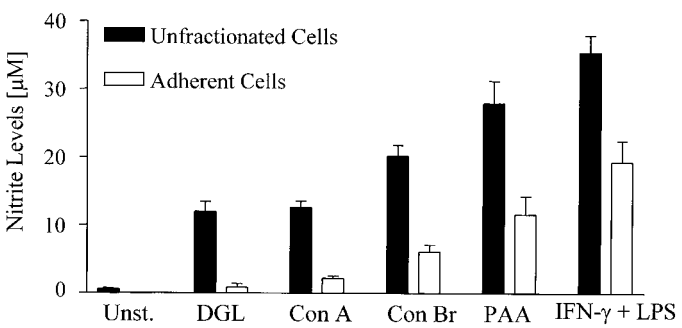

FIG. 3. Comparison of nitrite levels produced in unfractionated peritoneal cell cultures (closed bars) or peritoneal adherent cell cultures (open bars) after lectin stimulation. Cells were cultivated for 48 h and nitrite was determined by the Griess reaction. Each test was performed twice in triplicate. The bars represent the mean of replicates ± SD. Unst., unstimulated cells.

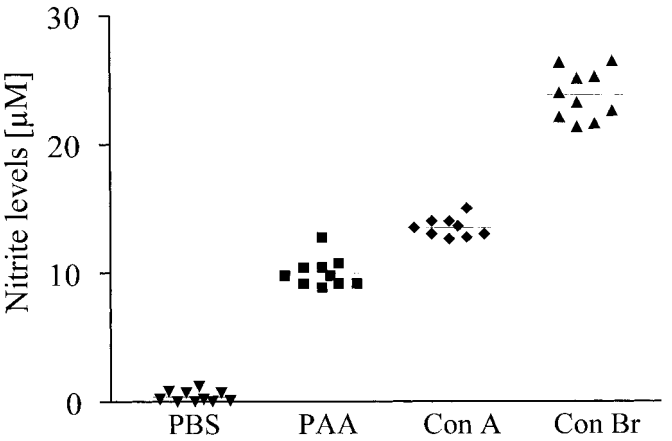

FIG. 4. *Ex vivo* assessment of lectin-induced NO production by murine peritoneal cells. Six h after lectin ip stimuli (100 μg) or PBS ip, a pool of peritoneal cells was collected and cultivated without further stimuli. Supernatants were tested for NO production at 48 h. The experiment was made with three animals per group. Each point represents one replicate of two different experiments. The horizontal line represents the mean.

NO blockage in vivo augmented effects of lectins. To investigate *in vivo* a possible effect of NO produced, we injected lectin in the left hind footpad and evaluated the number of cells in the draining popliteal lymph node. The contralateral footpads were injected with PBS and those corresponding lymph nodes were evaluated as an indication of unspecific changes. Groups of lectin injected animals were injected ip with aminoguanidine, a selective iNOS inhibitor, or PBS as control to investigate the NO involvement in the phenomenon. Lymph nodes from lectin-treated animals exhibited lower cellularity indexes than those treated with lectin + aminoguanidine (Fig. 5). After Con Br or PAA administration, the *in vivo* blockage of NO production increases ninefold (on average) the number of cells in draining lymph nodes, contrasted to unblocked controls, suggesting an involvement of lectins in NO production *in vivo* ($P \leq 0.01$).

DISCUSSION

Data presented here demonstrate that lectins can induce NO production by murine mononuclear cells. Lectins have a direct effect on macrophages but levels of NO increase significantly when adherent and nonadherent cells are cultivated together. It is noteworthy that *in vivo* lectin administration leads to NO production by peritoneal cells. Additionally, NO production *in vivo* seems to be implicated in reducing cell proliferation in lymph nodes draining the area of lectin injection. Therefore it is possible that NO mediates some of the immune system responses to lectins.

Structurally similar lectins differ in their capacity to induce proliferation and cytokine production in human

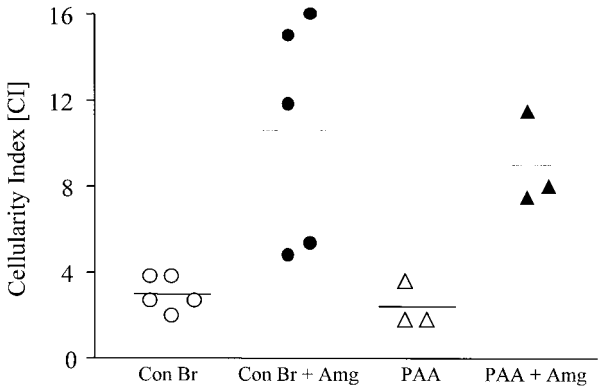

FIG. 5. Evaluation of lectin-mediated lymph node cells stimulation: Fifteen h after Con Br or PAA injection in the hind footpad the draining lymph nodes were collected and cell numbers were estimated. Results are shown as cellularity index (number of cells of lectin − draining lymph node divided by number of cells of PBS − draining contralateral lymph node). Circles represent animals stimulated with Con Br and triangles represent animals stimulated with PAA. Animals treated at 24 and 0 h before lectin stimuli with aminoguanidine (Amg), a seletive iNOS inhibitor, are represented by closed symbols and those treated with PBS are represented by open symbols. The line represents the mean of each group. (CI of PBS/PBS in aminoguanidine-injected animals was 0.8).

mononuclear cells (35). Recently it has been shown that human monocytes are able to produce low levels of NO when stimulated by pokeweed mitogen lectin (36). Our results extend these observations to NO production by murine mononuclear cells. Even highly similar lectins such as Con A and Con Br (4) differ in the pattern and intensity of stimulation of NO production. Other glucose–mannose lectins structurally similar to Con A were only capable of inducing low levels of NO (data not shown). In contrast, PAA lectin, which presents less structural homology with Con A among the tested lectins, was a potent stimulator of NO production, achieving levels similar to those obtained with LPS plus IFN-γ (gold standard for NO production). This may indicate differences in affinity to cell surface molecules (37, 38) or some degree of toxicity.

Observations made more than 25 years ago have led to the recognition that Con A can induce macrophage activation (39). More recently Con Br and DGL have also been shown to induce macrophage activation as measured by spreading and H_2O_2 production (40). Since Con A was found to bind directly on macrophages (41, 42) and TNF-α production by bone marrow macrophages was shown to be directly induced by the amebic gal-lectin (43), we investigated whether lectins could directly stimulate NO production by murine peritoneal macrophages. Lectin-stimulated adherent cells do produce NO, although nitrite levels are significantly lower than that observed in unfractionated mononuclear cell cultures. This suggests that besides the direct effect of lectins on macrophages, another mechanism such as lectin-induced lymphocyte NO production (12) or IFN-γ production (35), which would enhance macrophage NO production, is implicated in lectin-induced NO production. This also indicates that lymphocyte stimulation is probably an important mechanism following lectin treatment. It may be argued that contaminant lymphocytes remaining after washes could induce NO production in adherent cell cultures, although macrophages are reported to constitute above 90% of cell population in this assay (44).

Studies on *in vivo* effects of lectins on macrophages are rare. Welsch and Schumacher showed that Con A could bind to macrophages *in vivo* (45). Macrophage-dependent anti-parasitic effects were also observed after *in vivo* administration of Con Br (5). In the present report, nitrate/nitrite serum levels after lectin ip injection were undetectable. However, *ex vivo* experiments showed that cells from lectin-treated animals sustain *in vitro* NO production without further stimulation. These observations suggest that all tested lectins were able to stimulate peritoneal cells *in vivo.*

It is well-established that Con A induces lymphocyte proliferative responses (2) and a recent paper demonstrated that Con A, DGL, and Con Br were able to recruit cells to the peritoneal cavity (40). These lectins are also capable of inducing popliteal lymph node enlargement and increased cell counts 15 h after subcutaneous injection in mice footpads (46). Finally Con Br and DGL stimulation were found to involve activation proliferation of T-lymphocytes, assessed by elevated expression of CD25 and proliferating cell nuclear antigen in draining nodes (unpublished data). In contrast, NO may play a role in immunosuppressive effects (47, 48) like diminished proliferative responses (9, 49, 50), particularly of T helper 1 (12, 51). Mills showed that NO blockage *in vitro* leads to augmented Con A-induced rat splenic leukocyte proliferation and that macrophages were necessary for such effect (47). Additionally, Fecho and colleagues showed depressed Con A responsiveness of lymphocytes when macrophage NO production was induced (50). We therefore investigated here whether lectin-induced NO production would interfere with lectin effects *in vivo.* NO blockage has markedly increased cell numbers in stimulated draining lymph node compared to unblocked controls, suggesting that the lectin-induced NO production interferes with lymphocyte proliferation *in vivo.* These data also provide additional evidence that lectins are able to induce NO synthesis *in vivo.*

When administered orally as experimental vaccine carriers, lectins are able to bind to the gut mucosa and improve antigen uptake (52). Although lectins such as Con A are well-tolerated (53), in higher doses they elicit tissue injury by an unknown mechanism (54). Several lectins have been shown to stimulate TNF-α (43, 55) and IFN-γ production (35), and we currently show that lectins are also able to promote NO production by murine macrophages. This provides a further rationale for the investigation of lectins as putative adjuvants, improving anti-

gen delivery and modulating immune response. It is also of interest to determine whether lectin effects such as anti-tumoral and anti-parasitic capacity are mediated via the nitric oxide pathway.

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