Rodent and Human Mast Cells Produce Functionally Significant Intracellular Reactive Oxygen Species but Not Nitric Oxide*

In immunity, reactive oxygen species (ROS) and nitric oxide (NO) are important antimicrobial agents and regulators of cell signaling and activation pathways. However, the cellular sources of ROS and NO are much debated. Particularly, there is contention over whether mast cells, key secretory cells in allergy and immunity, can generate these chemical species, and if so, whether they are of functional significance. We therefore examined directly by flow cytometry the capacity of mast cells to generate intracellular ROS and NO using the respective cell-permeable fluorescent probes dichlorodihydrofluorescein and dianminofluorescein and evaluated the effects of inhibitors of ROS and NO synthesis on cell degranulation. For each of three mast cell types (rat peritoneal mast cells, mouse bone marrow-derived mast cells, and human blood-derived mast cells), degranulation stimulated by IgE/antigen was accompanied by production of intracellular ROS but not NO. Inhibition of ROS production led to reduced degranulation, indicating a facilitatory role for ROS, whereas NO synthase inhibitors were without effect. Likewise, bacterial lipopolysaccharide and interferon-γ over a wide range of conditions failed to generate intracellular NO in mast cells, whereas these agents readily induced intracellular NO in macrophages. NO synthase protein, as assessed by Western blotting, was readily induced in macrophages but not mast cells. We conclude that rodent and human mast cells generate intracellular ROS but not NO and that intracellular ROS but not intracellular NO are functionally linked to mast cell degranulation.

Mast cells are secretory cells central to specific and innate immunity, allergy, and inflammation (1–5). In specific IgE-mediated responses, they are activated by antigen to release chemical mediators such as histamine, proteases, prostaglandins, and cytokines (1), whereas in innate responses to bacteria, they promote neutrophil phagocytosis (3, 4) and lymph node hyperplasia (5) via the production of tumor necrosis factor. In keeping with a role in innate defense, mast cells can directly phagocytose and kill bacteria (6). Essential to bacterial killing by “professional” phagocytic cells such as neutrophils and macrophages is the production of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide, nitrogen oxide species such as nitric oxide (NO), and their combined product peroxynitrite (7–10). However, in mast cells, these pathways are less well characterized, and there is debate over whether mast cells generate biologically significant amounts of reactive oxygen and nitrogen species.

Several studies (11–13) have reported that rat peritoneal tissue type mast cells (RPMC) or mast cells of the rat RBL-2H3 cell line (14, 15) release ROS into the extracellular milieu in response to a range of stimuli. These studies measured ROS-induced light emission by scintillation counting (11) or using luminol or the luciferin-related compound 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroamidazo[1,2-e]pyrazine-3-one (12–15), and all of these techniques suffer from interference and lack of specificity (16). Using the highly sensitive real-time chemiluminescent probe Pholasin, we could not detect the release of ROS by purified rat tissue mast cells activated with IgE/antigen, phorbol myristate acetate, or ionomycin, whereas macrophages from the same starting populations of peritoneal cells readily released ROS at high levels (16). Because Pholasin is not cell-permeable, it detects released rather than intracellular ROS. Therefore, ROS may be generated but not exported. Indeed, intracellular ROS generation has been reported for RPMC (17–19), a mouse mast cell line (20), RBL-2H3 cells, and mouse bone marrow-derived mast cells (21). To date, no studies have been performed with human mast cells.

Reports from several groups claim that mast cells produce NO (22–26), although again, studies from our own laboratory (27–29) have provided contradictory evidence. Salvemini et al. (22) reported the release of a NO-like activity from rat peritoneal mast cells, although their preparations contained up to 15% macrophages, a known source of NO. Bidri et al. (23) reported expression of the inducible form of NO synthase (NOS2) and nitrite production by mouse bone marrow-derived mast cells, whereas Gilchrist et al. (24–26) have demonstrated inducible or constitutive NOS expression, nitrite production, and intracellular NO-induced DAF fluorescence in activated RPMC and the human tumor-derived HMC-1 and LAD2 mast cell-like lines. In contrast, we found that interferon (IFN)-γ stimulated or spontaneous NO production by rat or mouse peritoneal mast cells diminished as mast cells were progressively purified, such that even in preparations that contained

* This work was supported by a grant (to J. W. C.) from The Wellcome Trust and by National Institutes of Health intramural funds. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Laboratory of Allergic Diseases, NIAID, National Institutes of Health, Bldg. 10, Rm. 11C 209, Bethesda, MD 20892-1881. Tel.: 301-594-1276; Fax: 301-480-8384; E-mail: ejswindle@niaid.nih.gov.

‡ From the ‡Department of Pharmacology, University of Liverpool, Liverpool L69 3GE, United Kingdom and the §Laboratory of Allergic Diseases, NIAID, National Institutes of Health, Bethesda, Maryland 20892

§ The abbreviations used are: ROS, reactive oxygen species; NO, nitric oxide; NOS, NO synthase; AG, aminoguanidine; BMMC, mouse bone marrow-derived mast cells; HUMC, human mast cells; RPMC, rat peritoneal mast cells; DNP-HSA, dinitrophenylated human serum albumin; DPI, diphenyleleniodumium; DCF, dichlorodihydrofluorescein; FL1, fluorescence channel 1; FS, light forward scatter; SS, light side scatter; IFN, interferon; t-MMMA, N5-monomethyl-t-arginine; LPS, bacterial lipopolysaccharide; OVA, ovalbumin; SA, streptavidin; DAF, dianminofluorescein; DAF-2, 4,5-diaminofluorescein; ANOVA, analysis of variance.

Received for publication, August 24, 2004
98–99% mast cells, the NO production could be fully accounted for by the residual 1–2% non-mast cells (27, 28). Furthermore, mixed populations of macrophages and mast cells produced NO in response to IFN-γ only when the macrophages expressed the IFN-γ receptor (29). Therefore, at least in IFN-γ-driven responses, a small minority of contaminating cells can contribute substantially to nitrite production. In addition, we have consistently been unable to detect nitrite production by IgE/antigen-activated mast cells of various rodent phenotypes.2

In view of our demonstration that mast cells do not release ROS to the outside of the cell (16) and the apparent differences between our own and other groups relating to whether or not mast cells produce NO (22–29), the aims of the present study were as follows: 1) to examine directly, by flow cytometry employing specific and sensitive fluorescent intracellular probes, the capacity of rodent and human mast cells to generate intracellular ROS and NO; 2) to ascertain, using the appropriate pharmacological agents, the potential contribution of intracellular ROS and NO to mast cell secretory responses; 3) to investigate whether the capacity or inability of mast cells to generate ROS or NO relates to their species. We used exclusively tissue-derived or primary culture-derived mast cells rather than mast cells of tumor phenotype as used in many previous studies.

Our results show that activated tissue type or primary cultured rodent and human mast cells generate intracellular ROS. Inhibition of ROS production led to suppression of IgE/antigen-mediated degranulation in all mast cell types, indicating a functional link between these responses. However, activated rodent and human mast cells did not generate intracellular NO or express NOS2 protein, and inhibitors of NO synthesis were without effect on cell activation.

**EXPERIMENTAL PROCEDURES**

**Animals and Reagents**—Female Brown Norway rats (150–200 g) were obtained from Harlan Olac (Bicester, UK) and maintained in the university animal housing unit where food and water were provided ad libitum. Rats were sensitized by subcutaneous injection of ovalbumin (OVA/alum (2 × 0.2 ml, total dose of 80 µg of OVA, 8 mg of aluminum hydroxide) on days 0 and 7, and the rats were sacrificed humanely on day 14 by asphyxiation in CO2. Experimental procedures were approved by the University of Liverpool and were in accordance with guidelines set by the Home Office (London, UK).

Aluminum hydroxide, Dulbecco's modified Eagle's medium, diphenylenoendion (DPI), L-glutamine, bacterial lipopolysaccharide (LPS), OVA, mouse monoclonal anti-β-actin antibody, toluidine blue, trypan blue, sulphanilamide and N-(1-naphthyl)ethylenediamine dihydrochloride were purchased from Sigma. Fetal calf serum, gentamicin, and Hanks' balanced saline solution were from Invitrogen. 4,5-Diaminofluorescein (DAF-2) diacetate, dichlorodihydrofluorescein (DCF) diacetate, amino
guanidine (AG), and Nω-n-monomethyl-arginine (L-NMMA) were obtained from CN Biosciences (Nottingham, UK). Cytokines were obtained from Peprotech (Rocky Hill, NJ). Lysis buffer and SDS-polyacrylamide gels were from Invitrogen. Mouse monoclonal anti-NOS2 and rabbit anti-Lyn antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and rabbit polyclonal anti-NOS2 was from Calbiochem. Cell culture media and supplements were from Invitrogen or BIOSOURCE International (Camarillo, CA).

**Cell Isolation and Cultures**—Rat mast cells and macrophages were obtained by peritoneal lavage and purified by density gradient fractionation as described previously (16). Purified mast cell preparations contained >98% mast cells, whereas the macrophage preparations contained >85% monocyte/macrophages and <1% mast cells by metachromatic staining in 0.05% toluidine blue and by Giemsa staining.

---

2 E. J. Swindle and J. W. Coleman, unpublished observations.
of cytospin preparations. Mouse bone marrow-derived mast cells (BMMC) were grown from femoral marrow cells of BALB/c mice and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, 1.0 mM sodium pyruvate, non-essential amino acids, 0.0035% 2-mercaptoethanol, and 30 ng/ml mouse interleukin-3. Cells were used after 4–6 weeks of culture. Human mast cells (HUMC) were grown from CD34-positive peripheral blood mononuclear cells, obtained following informed consent. CD34-positive cells were cultured in interleukin-3 (week 1 only), stem cell factor, and interleukin-5 as described (30). HUMC were >95% pure by toluidine blue staining of cytospin preparations and were used after 8–10 weeks of culture.

Cell Degranulation and Nitrite Assays—Mast cells from OVA-sensitized rats, as well as BMMC or HUMC optimally sensitized with mouse IgE anti-DNP or biotinylated human IgE, respectively (both at 100 ng/ml for 24 h), were seeded at 10–50,000/well in 80 μl in a 96-well culture plate. Enzyme inhibitors (10 μM) were added for 1 h, the cells were challenged with 10 μg/ml of the appropriate antigen (OVA (1 μg/ml), DNP-HSA (100 ng/ml), or streptavidin (SA, 100 ng/ml), respectively), and degranulation was measured as the release of serotonin or β-hexosaminidase for HUMC or BMMC. Results are means ± S.E. from 3 to 5 separate experiments performed in duplicate. *, p < 0.05, **, p < 0.01, and ***, p < 0.001 for comparison with antigen alone by ANOVA followed by paired Student’s t test.

Immunoblots for NOS2 Protein—IgE-loaded BMMC and HUMC were incubated in complete medium with or without the appropriate antigen or LPS and IFN-γ under conditions that optimally induced cell activation measured as degranulation and ROS production. At various times (2–24 h), the cells were washed twice in serum-free medium, suspended at 2–24 h, and then mixed with an equal volume of boiling lysis buffer supplemented with 100 mM dithiothreitol, protease inhibitor mixture, and 2-mercaptoethanol as described (32). The cell extracts were boiled for 3 min and electrophoresed on 4–12% SDS-polyacrylamide gels. The separated proteins were then electrophoretically transferred to nitrocellulose membranes and probed with mouse monoclonal anti-NOS2 or rabbit polyclonal anti-NOS2. To monitor protein loading, blots were stripped and reprobed with monoclonal mouse anti-β-actin or rabbit anti-Lyn. Blots were developed with horseradish peroxidase-linked secondary antibody followed by chemiluminescent reagent and then exposed to x-ray film.

Data Presentation and Statistical Analysis—Data were analyzed by ANOVA followed by two-tailed Student’s t test. Differences were considered significant when p < 0.05.

RESULTS

Intracellular ROS Production and Relationship to Degranulation in Antigen-stimulated Mast Cells—Intracellular ROS production was monitored using the ROS-sensitive fluorescent probe DCF, and the effects of the flavoenzyme inhibitor DPI and the NOS inhibitor L-NMMA on both ROS production and cell degranulation were evaluated. In the case of RPMC, OVA produced a dramatic shift to the left of mast cell SS after 30 min.

**Fig. 2. Effect of DPI and L-NMMA on intracellular ROS production and degranulation.** Experiments were conducted as in Fig. 1, and results were averaged over 3–5 experiments. A–C show ROS generation, expressed as the percentage of antigen response, and D–F show degranulation for RPMC, HUMC, and BMMC, respectively. For degranulation experiments, RPMC (D), HUMC (E), or BMMC (F) were incubated for 1 h with L-NMMA or DPI prior to challenge with the appropriate antigen for 30 min. Degranulation was measured as net serotonin release for RPMC or β-hexosaminidase for HUMC or BMMC. Results are means ± S.E. from 3 to 5 separate experiments performed in duplicate. *, p < 0.05, **, p < 0.01, and ***, p < 0.001 for comparison with antigen alone by ANOVA followed by paired Student’s t test.
Mast Cells Generate Intracellular ROS but Not NO

Intracellular NO—Intracellular NO was measured by DAF-2 fluorescence in OVA- and LPS- and IFN-γ-stimulated RPMC, DNP-HSA-stimulated BMMC, and SA-stimulated HUMC, and the effects of NOS inhibitors on FL1 fluorescence were examined. In the first series of experiments, RPMC were incubated overnight with the NOS inhibitors l-NMMA or AG before incorporation of DAF-2 for 1 h. Cells were then stimulated with OVA under optimized conditions, and DAF-2 fluorescence and degranulation were monitored as described above. Following OVA stimulation for 0.5 h, the percentage of mast cells fully degranulated (upper left and lower left quadrants) increased from 3 to 18%, but there was only a negligible change in DAF-2 fluorescence (upper left and upper right quadrants) (Fig. 4, A and B). Furthermore, in contrast to ROS detection (Fig. 1C), OVA did not lead to the appearance of cells in the upper left quadrant, i.e. FL1-positive degranulating cells (Fig. 4B). In addition, the FL1 signal was not reversed by either l-NMMA (Fig. 4C) or AG (Fig. 4D). Histograms of DAF-2-dependent fluorescence confirmed that OVA produced no DAF-2 signal (Fig. 4E) and that l-NMMA (Fig. 4F) and AG (Fig. 4G) were without effect.

Similar responses were observed for BMMC or HUMC. Stimulation with the appropriate antigen produced no DAF-2 signal (Fig. 4, H and K, respectively), and l-NMMA or AG had no effect (Fig. 4, I–J and L–M). The unresponsiveness of all types of antigen-stimulated mast cells with respect to DAF-2 fluorescence was in sharp contrast to rat macrophages. When these cells were stimulated with LPS and IFN-γ, a dramatic shift in DAF-2 fluorescence was observed that was fully inhibited by both l-NMMA and AG (Fig. 4, N–P).

Averaged data across experiments confirmed that OVA, SA, or DNP-HSA did not induce NO production (elevated FL1) within 30 min of stimulation of RPMC, HUMC, or BMMC, respectively, and accordingly, l-NMMA and AG were without effect (Fig. 5, A–C). In RPMC, later time points of 1, 7, and 24 h after OVA stimulation were also investigated and produced similar results showing a lack of NO production over 24 h. In the same experiments, OVA did not induce the release of NO detectable as nitrite (Table I). The unresponsiveness of mast cells to antigen was in sharp contrast to rat macrophages, which showed a marked increase in DAF-2 fluorescence following stimulation with LPS and IFN-γ, which was fully inhibited by both l-NMMA and AG (Fig. 5D).
Intracellular NO Generation by Macrophages but Not Mast Cells Stimulated with LPS and IFN-γ—Since RPMC did not produce intracellular NO in response to OVA, we next examined whether they might respond to the classical NOS2 activating agents LPS and IFN-γ, although these agents do not induce mast cell degranulation. As for antigen, LPS and IFN-γ induced no DAF-2 signal in RPMC (Fig. 6A) but induced a strong signal in macrophages (Fig. 6B). Accordingly, L-NMMA and AG blocked the macrophage DAF-2 signal (Fig. 6B) but were without effect on RPMC fluorescence (Fig. 6A). Data averaged across experiments confirmed these results (Fig. 6, C and D). Additionally, extracellular nitrite was measured in LPS- and IFN-γ-stimulated macrophages and LPS and IFN-γ or OVA-stimulated RPMC and gave comparable results to those obtained in the DAF-2 fluorescence experiments (Table I).

Expression of NOS2 Protein by Macrophages but Not Mast Cells—Incubation of IgE-sensitized BMCC or HUMC with antigen or OVA failed to stimulate the expression of NOS2 protein as assessed by immunofluorescence microscopy (data not shown).
to induce cellular NOS2 protein detectable with either the monoclonal (Fig. 7) or the polyclonal antibody, whereas on each gel, NOS2 protein was readily detected in a positive control extract from rat macrophages stimulated with LPS and IFN-γ.

**DISCUSSION**

In this study, we show that primary cultured mast cells of three different species (rat peritoneal tissue type, mouse bone marrow-derived, and human CD34+ blood cell-derived) generate intracellular ROS when activated by IgE/antigen but do not produce intracellular NO when activated by either IgE/antigen or LPS and IFN-γ. In each case, cells were activated by antigen under conditions that gave the optimal release of granule mediators. LPS and IFN-γ readily induced NO in control cells (rat macrophages). Furthermore, antigen or LPS and IFN-γ failed to elicit expression of the inducible form of NO synthase (NOS2) in mouse and human mast cells. Intracellular ROS production by mast cells was inhibited by the flavoenzyme inhibitor DPI, whereas macrophage NO production was inhibited by the NOS inhibitors L-NMMA or AG, confirming the identity of these products.

We reported recently that activated rat peritoneal tissue type mast cells do not release ROS detectable by the sensitive real-time chemiluminescent probe Pholasin (16). In the present study, these same cells are shown to generate ROS intracellularly. Thus, ROS generated intracellularly by mast cells are not exported to the outside of the cell. In this respect, mast cells differ from activated macrophages that readily export high levels of ROS (16). Mast cells contain intracellular mechanisms for neutralization or chemical conversion of ROS including intracellular superoxide dismutase and glutathione, levels of which are enhanced following mast cell activation (17), and presumably, these are sufficient to breakdown ROS before they can be exported.

**TABLE I**

Nitrite levels (μM) in supernatants from mast cells and macrophages

Mast cells or macrophages were cultured as described with or without either L-NMMA (500 μM) or AG (100 μg/ml) before stimulation with either OVA (1 μg/ml, mast cells only) or IFN-γ (100 ng/ml) + LPS (10 μg/ml) and nitrite determined in cell supernatants after 24 or 48 h. Results are means ± S.E. from three separate experiments. *, p < 0.01 for comparison with stimulated cells by ANOVA followed by paired Student’s t test with Bonferroni correction. ND = not detected, assay limit was 1 μM.

<table>
<thead>
<tr>
<th>Mast Cells</th>
<th>LPS + IFN-γ</th>
<th>LPS + IFN-γ</th>
<th>LPS + IFN-γ</th>
<th>LPS + IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVA</td>
<td>24 h</td>
<td>24 h</td>
<td>48 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Unstimulated</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2.4 ± 0.8*</td>
</tr>
<tr>
<td>Stimulated</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>9.8 ± 2.0</td>
</tr>
<tr>
<td>Stimulated + L-NMMA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Stimulated + AG</td>
<td>ND</td>
<td>ND</td>
<td>2.3 ± 0.7*</td>
<td>7.1 ± 0.7*</td>
</tr>
</tbody>
</table>

**FIG. 5. Effect of L-NMMA and AG on intracellular DAF-2 fluorescence.** DAF-2 fluorescence by antigen-stimulated RPMC (A), HUMC (B), and BMCC (C) and by LPS and IFN-γ-stimulated rat macrophages (D) was measured as in Fig. 4, and results were presented as means ± S.E. for 3 experiments performed in duplicate. **, p < 0.01 and ***, p < 0.001 for comparison with LPS and IFN-γ alone by ANOVA followed by paired Student’s t test.
In our experiments, the IgE/antigen-induced production of intracellular ROS was accompanied by 20–50% release of granule-associated mediators in all three types of mast cell studied. To examine the potential regulatory role of intracellular ROS in mast cell activation, we blocked ROS production using the flavoenzyme inhibitor DPI. This agent blocked completely antigen-induced ROS production by all mast cell types. Concurrently, DPI inhibited granule-associated mediator release by 80% for RPMC and HUMC but by 15% for BMMC. We conclude that the production of ROS in mast cells is functionally linked to degranulation in RPMC and HUMC but less so in BMMC. However, these processes may not necessarily be interdependent since under certain conditions, for example combinations of diethylidithiocarbamate with calcium ionophore or compound 48/80 (33) or at subthreshold concentrations of antigen or substance P (19), the mast cell production of ROS can be dissociated from amine release. However, studies with the rat RBL-2H3 mast cell line, as in the present study, reveal a positive correlation between inhibition of ROS production and inhibition of mast cell degranulation (14, 15, 21). Inhibition of RBL-2H3 cell ROS production and degranulation by DPI is accompanied by decreased elevations of cellular calcium and reduced activation of phospholipase Cγ and the linker for activation of T cells, implicating signaling events prior to calcium mobilization as ROS targets (21). DPI inhibits flavoenzymes other than NADPH oxidase including mitochondrial complex I (34–36), which leads in turn to inhibition of mitochondrial-associated ROS production (37). There is an increasing body of evidence that mitochondrial-derived ROS are not simply by-products of respiration but may act as signaling molecules in normal cell function (38). Therefore, the DPI-dependent inhibition of either ROS production and/or degranulation observed within mast cells may occur at the mitochondrial level.

Our previous study (16) and others (39, 40) reported that cell- or chemical-derived hydrogen peroxide (a ROS generated from superoxide) inhibits antigen-driven mast cell mediator release. Thus, there is a potential dual role for ROS in mast cell activation; exogenous hydrogen peroxide, generated for example by macrophages at high levels, inhibits mast cell activation, whereas low level endogenously generated ROS may be facilitatory for exocytotic degranulation.

There is debate over whether mast cells are capable of generating NO, and if so, whether the radical exerts autoregulatory effects on mast cell activation (22–29). Our own studies (27–29) failed to confirm reports from others (22–26) that mast cells generate NO. We found that IFN-γ-induced nitrite production by mixed mast cell/macrophage populations was almost entirely abolished as mast cells were purified, such that even in preparations that contained 98–99% mast cells, the NO production could be fully accounted for by the residual 1–2% macrophages (27, 28). Furthermore, mixed populations of macrophages and mast cells produced NO only when the macrophages expressed the IFN-γ receptor (29). Therefore, nitrite production and NOS2 mRNA detected by reverse transcriptase-PCR (24) could be of non-mast cell origin even in highly purified mast cell populations. In the current study, we show that mast cells of three species, rat peritoneal tissue type, mouse bone marrow-derived, and human CD34+ blood cell-derived, do not generate...
Our results and conclusions differ markedly from those of Gilchrist et al. (24–26) who, also using the intracellular NO probe DAF, reported NO production by RPMC (25) and human HMC-1 and LAD2 mast cells (26). In the case of RPMC, activation by IgE/anti-IgE led to NO production in a minority subset of cells that showed no visible signs of degranulation, whereas degranulating cells failed to generate NO. Interestingly, the NO-producing non-degranulating cells eventually did show degranulation, and this was interpreted as NO delaying mediator release (25). Our current results, showing a complete absence of NO production in IgE/anti-IgE-activated degranulating and non-degranulating RPMC over a wide range of time periods, cast doubt over the conclusions of Gilchrist et al. (24–26), or at least suggests that the responses of outlying cells does not reflect large populations as a whole. In their study, cell fluorescence was measured on limited numbers of cells (8–15 experiment), whereas in our experiments, we monitored NO production simultaneously with degranulation and found no differences in distribution of DAF signal between activated and non-activated mast cells in large populations of 10,000 cells. In addition, we detected no outlying high DAF-2 fluorescing cells, as would be predicted from the studies of Gilchrist et al. (25). Furthermore, although Gilchrist et al. (25) showed that inhibition of synthesis of the NOS co-factor biotin led to enhanced RPMC degranulation, our work (Refs. 27 and 28 and the present study) shows that direct inhibition of NO synthesis does not influence mediator release by rat, mouse, or human mast cells. Convincingly, Gilchrist et al. (26) demonstrated constitutive NOS protein expression and calcium-driven NO production in human HMC-1 and LAD2 mast cells and found that NO donors and inhibitors, respectively, inhibited and enhanced leukotriene production. Thus, in these mast cell lines, a calcium-driven, regulatory constitutive NOS-dependent NO response occurs. However, it should be stressed that the primary phenotype of HMC-1 and LAD2 cells is that of tumor cells. In the present study, using tissue type, bone marrow-, or blood-derived mast cells of rodent and human origin, we were unable to detect NO production under a range of conditions.

In conclusion, our results show that activated rat, mouse, and human mast cells generate intracellular ROS but not NO. In each species of mast cell studied, endogenous ROS were functionally linked to mast cell activation and may act to facilitate mediator release. With regard to NO, exogenous sources such as macrophages or epithelial cells are more likely than endogenous NO to be involved in regulation of mast cell activation and mast cell-dependent immune and allergic reactions.

REFERENCES
