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Attenuated Salmonella Vaccine-Induced Suppression of Murine Spleen Cell Responses to Mitogen Is Mediated by Macrophage Nitric Oxide: Quantitative Aspects

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Previous reports from our laboratory have shown that 7 days after infection of C3HeB/FeJ mice with an attenuated strain of *Salmonella typhimurium*, there is profound suppression of responses to B- and T-cell mitogens and suppression of the capacity of spleen cells to mount a primary, in vitro plaque-forming-cell (PFC) response to sheep erythrocytes. Inhibition of the PFC response was shown to be mediated by nitric oxide (NO), as N^G-monomethyl-L-arginine (NMMA) gave complete reversal of suppression. The experiments reported here examined the role of NO in suppression of the response to the mitogen concanavalin A (ConA). In contrast to the PFC system, it was found that addition of NMMA to ConA-stimulated immune spleen cells resulted in less than 20% reversal of suppression. However, addition of NMMA resulted in a 50% reversal of suppression in cocultures of immune and normal spleen cells at a ratio of 1:4. A complete restoration of ConA-induced responses was achieved in cocultures incubated in medium containing a reduced concentration of L-arginine plus 1.25 mM NMMA. Investigation of why NMMA alone was not 100% effective in reversing suppression showed that addition of ConA significantly augmented production of nitrite and gamma interferon (IFN-γ) in cocultures containing immune cells. Addition of anti-IFN-γ reduced nitrite levels in the cultures, although results with the combination of anti-IFN-γ and NMMA were not significantly better than results with NMMA alone. These findings suggest that suppression in cultures stimulated with ConA is difficult to reverse completely with NMMA alone because of an overproduction of NO, which can be offset by either reducing the L-arginine concentration or blocking IFN-γ. The quantitative relationship between nitrite levels and suppression in cocultures was examined. It was found that suppression did not correlate directly with the nitrite concentration but rather with the $\log_{10}$ of the nitrite concentration. Nitrite levels above 15 μM gave almost complete suppression, and levels between 1 and 10 μM gave a wide range of suppression. These results strongly support NO as the suppressor factor in *Salmonella*-induced immunosuppression of responses to ConA and, by inference, suppression of responses to mitogens induced by other microbes. The results show that involvement of NO cannot always be demonstrated by simple addition of NMMA to suppressed mitogen-stimulated spleen cell cultures.

Macrophage-mediated immunosuppression has been reported after infection by a variety of microbes, including bacteria, fungi, and parasites (14). Many of these organisms, such as various mycobacteria (23, 30, 38), *Rickettsia tsutsugamushi* (22), *Toxoplasma gondii* (36), *Trypanosoma cruzi* (9), and *Histoplasma capsulatum* (10, 29), are intracellular pathogens of macrophages. Some nonviable microbes, such as *Corynebacterium parvum* (32, 39), and microbial extracts, such as peptidoglycan (13), also induce suppressor macrophages. For some viable microbes or nonviable stimulators, the macrophage-derived suppressor factor was identified as prostaglandin or peroxide, but for others, the suppressor factor was not indomethacin or catalase inhibitable (14, 27). In 1991, macrophage-derived nitric oxide was shown to mediate suppression of responses to mitogens in normal or *C. parvum*-stimulated rat spleen cell cultures (3, 28) and in mouse concanavalin A (ConA)-stimulated spleen cell cultures suppressed by addition of normal peritoneal macrophages (1, 28).

We had observed that inoculation with an attenuated *aroA Salmonella typhimurium* strain, SL3235, induced excellent protection against virulent salmonella or listeria challenge as well as profound immunosuppression of responses to mitogen stimulation in the spleen (17, 24) and inability of splenocytes to make an in vitro antibody plaque-forming-cell (PFC) response to sheep erythrocytes (4, 6, 15). Using a temperature-sensitive attenuated strain of *S. typhimurium*, Deschenes et al. confirmed that infection induced suppression of responses to mitogens (11). Our study implicated macrophages in the suppressed responses to mitogen stimulation, as removal of adherent cells partially alleviated suppression (24). In the PFC response, we showed that both mature macrophages and macrophage precursors were suppressor cells (4, 5). Further, we found that macrophage-derived nitric oxide was the suppressor factor responsible for inhibition of the PFC response, since the competitive inhibitor of NO synthesis N^G-monomethyl-L-arginine (NMMA) completely reversed suppression (7, 16). NMMA has also been shown to block splenic suppression induced by *Trypanosoma brucei* (31, 34) and *Toxoplasma gondii* (8), although in contrast to our results assessing PFC responses, only partial reversal of suppression of ConA-stimulated responses was achieved.

In this study, we investigated the role of NO in our previously observed suppression of responses to ConA in spleen cells from SL3235-immunized mice (24). Surprisingly, the maximal reversal of suppression achieved with NMMA alone was less than 20% of control values at optimal NMMA concentrations and there did not appear to be a good correlation be-

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tween the degree of suppression and NO levels. These results could have led to the conclusion that NO is not the suppressor factor. However, further dissection of the system showed that complete reversal of suppression of responses to ConA could be achieved by coculturing the immune cells with normal cells, partially depleting L-arginine in the culture medium, and adding NMMA. Suppression was found to be a function of the $\log_{10}$ of the nitrite concentration, accounting for what initially appeared to be a lack of correlation between NO levels and immune dysfunction. These experiments were important, as they explored the quantitative relationships between NO levels and immunosuppression and showed that when suppression is assessed in ConA-stimulated spleen cells, failure of NMMA to reverse suppression needs to be interpreted with caution.

MATERIALS AND METHODS

Mice. Female C3HeB/FeJ mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Animals were housed in sterilized plastic cages with Absorb-Dri for bedding. Mouse Chow (Purina, St. Louis, Mo.) and fresh water were available ad libitum. All mice were acclimatized for at least 1 week prior to being used at 8 to 12 weeks of age.

Bacterial strain. *S. typhimurium* SL3235, kindly provided by Bruce A. D. Stocker (Stanford University School of Medicine, Stanford, Calif.), is a smooth, avirulent strain (20) with an LD_{50} of greater than 10^7 bacteria when given intraperitoneally to C3HeB/FeJ mice. Log-phase cultures of SL3235 were prepared and used for immunization as previously described in detail (24). Mice were immunized intraperitoneally with 5×10^5 live bacteria in 0.5 ml of saline. Control mice received 0.5 ml of saline.

Reagents. NMMA was purchased from Calbiochem-Behring Corp. (La Jolla, Calif.). ConA was obtained from Sigma (St. Louis, Mo.). Neutralizing hamster immunoglobulin G monoclonal antibody to murine gamma interferon (IFN-γ) was obtained from Genzyme (Boston, Mass.).

Culture medium. The standard medium used for mitogenicity assays was powdered RPMI 1640 from GIBCO (Grand Island, N.Y.), to which was added L-glutamine, 100 U of penicillin per ml, and 100 mg of streptomycin per ml. In some mitogenicity assays, RPMI 1640 containing a reduction of the normal concentration of L-arginine (200 μg/ml) was used. For these experiments, medium was reconstituted with an RPMI 1640 Select-Amine kit (GIBCO). Serum was not used in the mitogenicity assays.

Cell preparation. Seven days after injection with *S. typhimurium* or saline, spleens were removed and teased gently into single-cell suspensions in cold RPMI 1640 and prepared as previously described (24). In some experiments, splenocytes from SL3235-injected mice were fractionated into adherent and nonadherent populations by adding 3.2×10^7 cells in 8 ml of RPMI 1640 with 5% fetal bovine serum (HyClone, Logan, Utah) to plastic petri dishes (100 by 15 mm, no. 1029; Falcon, Lincoln Park, N.J.) and allowing them to adhere for 2 h at 37°C. Nonadherent cells were removed by washing the plates two times with warm medium containing fetal bovine serum. Adherent cells were then gently scraped off. All cells were counted with a Coulter Counter (Coulter Electronics, Hialeah, Fla.) and adjusted to the proper cell concentrations. The viability of cells was routinely >95% as determined by trypan blue dye exclusion.

Mitogenicity assay. Cells were added in a volume of 100 μl of RPMI 1640 without serum to flat-bottomed, 96-well Costar (Cambridge, Mass.) plates. Normal or immune spleen cells were added at either 8×10^5 or 1.6×10^6 cells per 100 μl. For cocultures, graded doses of normal cells, immune cells, or immune adherent cells were added to 8×10^5 normal unfractionated spleen cells in a final volume of 100 μl per well. In most coculture experiments, 2×10^5 immune cells were added to 8×10^5 normal cells. ConA (0.1 μg per well), was added in 50 μl of RPMI 1640. NMMA or anti-IFN-γ was added to wells in 50-μl volumes. All wells were brought to a final volume of 200 μl with medium. Cells were incubated for 42 h in 5% CO_2, and [^{3}H]thymidine (0.5 μCi in 50 μl) was added. Six hours later cells were harvested with an Inotech (Southampton, Pa.) multichannel harvester. Thymidine uptake was determined by counting filters that were placed in CytoScint (Irvine, Calif.) liquid scintillation solution with a β-counter (Packard, Downers Grove, Ill.). Data are from triplicate cultures and are expressed as mean counts per minute ± standard deviations (SD) corrected for background values by subtraction of counts per minute in the absence of mitogen. In some experiments, suppression of ConA responses in immune groups was expressed as percent suppression compared with ConA-stimulated control cells by the formula percent suppression = [1 − (cpm of immune cells/cpm of normal cells)] × 100%. Cell viability was assessed by trypan blue exclusion. After 48 h in culture, cells were still at least 75% viable by this criterion.

Measurement of NO_2^- accumulation. Cell-free supernatants from spleen cell cultures were assayed for nitrite (NO_2^-) by the colorimetric Griess reaction as a measure of NO production. Briefly, 100-μl aliquots of supernatant were mixed with equal volumes of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2% H_3PO_4; Sigma) and incubated at room temperature for 10 min. The A_{550} was measured in an automated microplate reader (Bio-Rad, Richmond, Calif.). NO_2^- was quantitated with $NaNO_2$ (Sigma) as a standard.

IFN-γ production. IFN-γ levels in supernatants of splenocyte cultures were assessed by a sandwich enzyme-linked immunosorbent assay with a pair of anti-murine-IFN-γ antibodies purchased from PharMingen (San Diego, Calif.). The assay was carried out according to the manufacturer's recommendations. Murine IFN-γ, also from PharMingen, was used to construct the standard curve.

Statistics. The significance of the differences observed was assessed by Student's *t* test. Differences were considered significant when *P* values of <0.05 were obtained. Correlations between $\log_{10} NO_2^-$ concentration and immunosuppression were calculated by linear regression and expressed as r^2.

TABLE 1. Partial reversal of suppression of responses to mitogen by addition of NMMA to spleen cells taken from SL3235-immunized mice

Expt	NMMA (mM)	Mean cpm ± SD[a]		% Reversal of suppression	Mean nitrite production (μM) ± SD
		Normal	Immune		
1	0	47,044 ± 3,369	0	0	68.7 ± 1.6
	0.5	49,074 ± 8,185	2,748 ± 391	5.6	30.0 ± 1.5
	1.0	50,244 ± 1,376	6,643 ± 480	13.2	22.9 ± 0.3
	2.0	57,096 ± 677	9,611 ± 1,318	16.8	14.1 ± 0.6
2	0	60,023 ± 1,356	0	0	74.6 ± 2.3
	0.5	71,433 ± 2,735	5,233 ± 614	7.3	36.6 ± 1.6
	1.0	72,099 ± 125	10,978 ± 1,435	15.2	28.7 ± 1.2
	2.0	75,432 ± 3,565	8,912 ± 1,173	11.8	18.4 ± 0.9
3	0	92,702 ± 19,395	0	0	80.5 ± 3.1
	0.5	98,037 ± 5,853	86 ± 30	0.1	43.2 ± 1.7
	1.0	84,837 ± 8,990	9,320 ± 2,000	11.0	34.4 ± 1.0
	2.0	91,144 ± 20,333	11,921 ± 3,541	13.1	22.7 ± 1.4

[a] Responses of 8×10^5 normal or immune spleen cells from triplicate cultures.

RESULTS

Partial reversal of suppressed ConA responses by NMMA added into immune spleen cell cultures. Spleen cells harvested 7 days after SL3235 inoculation showed a dramatic suppression of T-cell proliferation to ConA compared with cells from uninfected mice (Table 1). In three experiments, NMMA at concentrations from 0.5 to 2.0 mM was only minimally effective in reversing suppression. While reversal by NMMA was statistically significant, the maximal responses achieved were less than 17% of the normal response. Examination of nitrite levels showed that NMMA inhibited nitrite production in a dose-dependent manner to a maximal level that was approximately 75% of control values at the highest dose tested (2 mM). No linearity was observed between the percent reversal of suppression by NMMA at various doses and the percent lowering of the nitrite levels. This apparent discrepancy in the quantitative relationships between nitrite and suppression are explored further in the experiments to be described below.

Proliferative response of normal cells to ConA is suppressed by coculture with immune spleen cells or immune adherent spleen cells. Graded doses of unfractionated or adherent spleen cells from immunized mice were cocultured with 8×10^5 normal spleen cells. A dose of unfractionated or adherent spleen cells at a concentration of 2×10^5 (a ratio of 1:4) gave almost 100% inhibition of the response of normal spleen cells to ConA (Fig. 1A). Inhibition was not due to cell crowding, as addition of equal numbers of normal spleen cells to 8×10^5 normal spleen cells had no effect (see Fig. 2). Adherent cells were as active as unfractionated cells in inducing suppression (Fig. 1B), supporting the hypothesis that mature macrophages are the source of the suppressor factor. Examination of nitrite production showed that cocultures containing either unfractionated spleen cells or adherent cells made substantial quantities of NO as measured by nitrite accumulation (Fig. 1A and

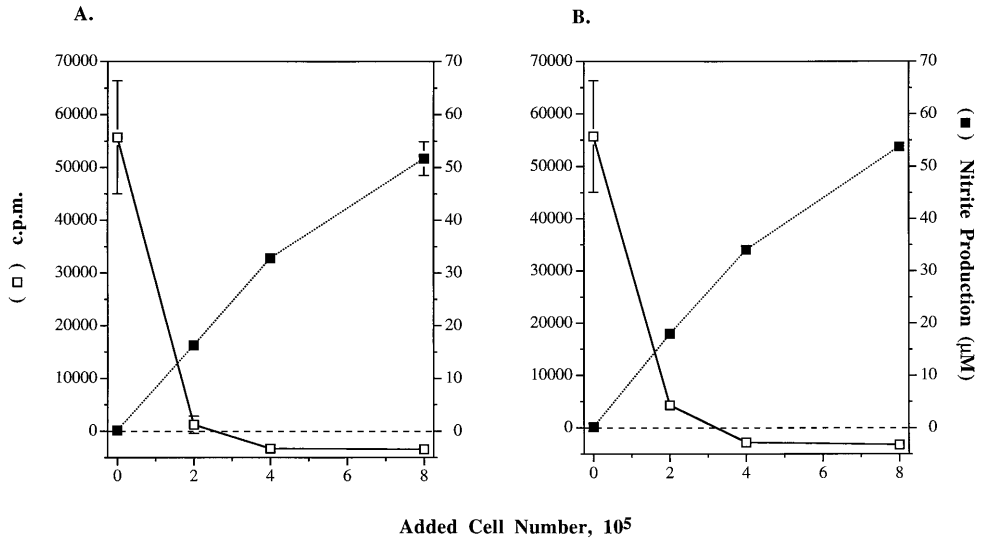

FIG. 1. Responses to mitogen and nitrite production in cocultures of normal spleen cells (8×10^5 cells per well) with addition of graded doses of either unfractionated immune spleen cells or immune adherent cells. [^{3}H]thymidine uptake (—□—) and nitrite production (—■—) are shown. (A) Cocultures of normal cells plus graded doses of unfractionated immune spleen cells. (B) Cocultures of normal cells plus graded doses of immune adherent cells. Data represent means ± SD of triplicate cultures from one of three similar experiments.

B). Increasing numbers of immune cells in the cocultures resulted in a dose-dependent increase in nitrite levels. However, levels of nitrite greater than 15 μM resulted in 100% suppression.

NMMA partially reverses suppressed ConA responses and reduces NO levels in cocultures. The same doses of NMMA that were tested on immune spleen cell cultures and found to be relatively ineffective in reversing suppression (Table 1) were used to treat cocultures of normal and immune spleen cells. The addition of NMMA, at all doses tested (0.5 to 1.25 mM), resulted in a 50% reversal of the suppressed response to ConA (Fig. 2). A steep drop in nitrite levels was observed at an NMMA concentration of 0.5 mM. Increasing the dose of NMMA further decreased nitrite levels to near baseline levels, but suppression was not correspondingly further reversed. NMMA did not significantly potentiate the normal response to ConA (Fig. 2) but in doses above 2 mM, inhibited normal responses (data not shown). A dose of 1.25 mM NMMA was used to test for inhibition of NO in cocultures of normal and immune cells. As shown in Table 2, normal cells, with or without ConA, did not make nitrite. Cocultures of normal plus immune cells, at concentrations of immune cells above 2×10^5, made detectable nitrite, and addition of ConA augmented nitrite production by more than 20 μM for immune cells in cocultures. While NMMA at 1.25 mM dramatically lowered nitrite levels in unstimulated cultures and in cultures given ConA, it was unable to completely block nitrite production when nitrite levels were above 20 μM.

Anti-IFN-γ partially restores responsiveness to ConA. The combination of IFN-γ plus lipopolysaccharide (LPS) is known to be one of the major inducers of inducible nitric oxide synthase (1, 12, 25, 35). We considered the hypothesis that ConA might stimulate lymphocytes to make IFN-γ, which in the presence of *Salmonella* LPS might lead to augmented NO production. Table 3 shows that normal spleen cells made no IFN-γ, but ConA stimulated its production. Cultures containing immune cells without addition of ConA had a moderate elevation of IFN-γ levels above that of controls. ConA treatment of immune cells or of cocultures with immune cells resulted in an approximately 10-fold increase in levels of IFN-γ. Anti-IFN-γ was tested alone and in combination with NMMA for the ability to reverse suppression and NO production. Anti-IFN-γ and NMMA alone were each effective in partially reversing suppression (Table 4). When used together, NMMA plus anti-IFN-γ restored responses to 74% of controls levels; however, the combination treatment was not statistically more

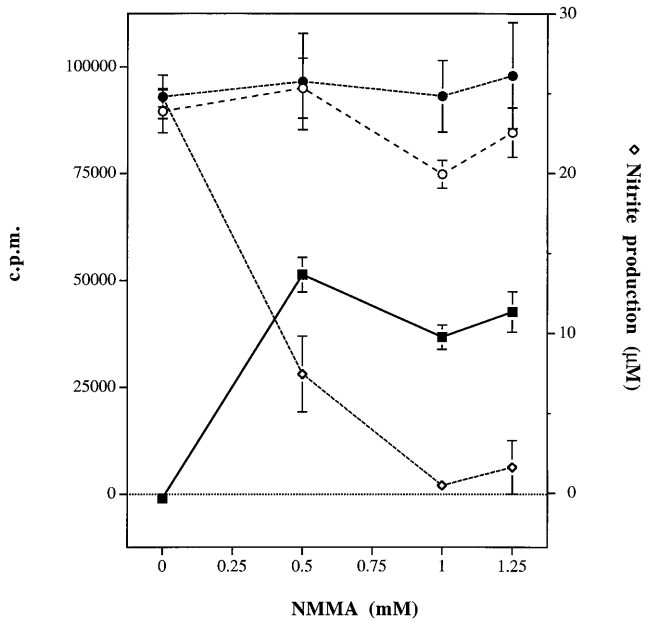

FIG. 2. Partial reversal of suppression of ConA-induced responses by NMMA. Normal spleen cells at concentrations of 8×10^5 (----○----) or 1×10^6 (----●----) cells per well or cocultures of 8×10^5 normal and 2×10^5 immune spleen cells per well (—■—) were incubated with graded doses of NMMA in the presence of ConA. Nitrite concentrations (----◇----)are shown for cocultures. [^{3}H]thymidine uptake was assessed 48 h after culture initiation. Data represent means ± SD of triplicate determinations from one of two similar experiments.

TABLE 2. Effect of NMMA on NO production by cocultures of normal and immune spleen cells

No. of cells (10^5) and cell type		Mean nitrite production (μM) ± SD with[a]:				% Inhibition of NO production with ConA plus NMMA versus ConA alone
Normal	Immune	No treatment	NMMA[b]	ConA	ConA plus NMMA	
8		0	0	0.2 ± 0.2	0.1 ± 0.2	
8	2	0 ± 0.1	0	24.7 ± 0.6	1.6 ± 1.7	93.5
8	4	2.4 ± 0.2	0	36.7 ± 3.9	5.2 ± 1.7	85.8
8	8	22.1 ± 4.1	1.8 ± 1.6	59.3 ± 3.8	11.8 ± 0.8	80.1

[a] Representative of three experiments.
[b] NMMA concentration = 1.25 mM.

TABLE 3. Augmentation of IFN-γ by addition of ConA to spleen cell cultures

Cell culture[a]	Mean IFN-γ production (pg/ml) ± SD after 48 h in culture[b]	
	Without ConA	With ConA
Normal	0	508 ± 186
Immune	137 ± 17	1,226 ± 151
Normal plus immune	225 ± 18	2,902 ± 117

[a] Normal and immune cell cultures contained 8×10^5 normal or immune cells per well. Cocultures contained 8×10^5 cells of each type per well.
[b] Representative of three experiments.

effective than NMMA alone. Higher levels of anti-IFN-γ (up to 600 ng/ml) were tested but gave no additional reversal of suppression (data not shown). Nitrite production decreased roughly in parallel to reversal of suppression, but a firm quantitative relationship between the percent reversal of suppression and the percent decrease in nitrite was not found.

Partial L-arginine depletion combined with addition of NMMA completely reverses suppression of responses to ConA. Since NO is made by macrophages from L-arginine (18, 19, 26), another approach to inhibiting NO synthesis is to decrease the L-arginine concentration in the medium (2). In the complete absence of L-arginine, normal splenocytes failed to proliferate in response to ConA (data not shown). However, partial depletion of L-arginine still supported responses of spleen cells from normal mice to the mitogen ConA, even in the presence of 1.25 mM NMMA (Fig. 3). When NMMA (1.25 mM) was added to cocultures containing immune cells in medium with standard amounts of L-arginine (200 μg/ml), an approximately 50% reversal of suppression was observed. Reduction of the arginine concentration to 50 or 25 μg/ml fully restored ConA responses to normal levels (Fig. 3A). The combination of NMMA and reduced arginine was able to completely inhibit nitrite production (Fig. 3B). In this experiment, the nitrite level observed for cultures that had only NMMA and a normal arginine concentration was detectable but low.

Correlation between degree of suppression of responses to mitogen and nitrite levels. In most of the preceding experiments there was an apparent nonlinearity between the nitrite concentrations and the percentages of suppression. A plot of percent suppression versus nitrite concentration for all of the experiments is presented in Fig. 4. The graph shows that the percent suppression is a function of the $\log_{10}$ of the amount of nitrite which accumulates in the medium. Thus, nitrite concentrations between 15 and 80 μM gave statistically indistinguishable levels of suppression between 80 and 100%. Similarly, at nitrite concentrations between 1 and 10 μM, large differences in percent suppression were observed for small changes in nitrite levels. Nitrite levels needed to be reduced to below 1 μM to obtain a 75% restoration of responses to mitogen.

DISCUSSION

The hypothesis that NO is the suppressor factor in microbe-induced immunosuppression in experimental infection models using mice was previously tested by us in a PFC assay using salmonella-infected animals. The complete reversal of inhibition of the PFC response obtained by adding NMMA to immune spleen cell cultures containing sheep erythrocytes as antigens provided evidence that NO was the suppressor factor (7, 16). For the experiments described in the present paper, we attempted to use NMMA to reverse suppression of ConA-induced responses in spleen cells taken from salmonella-infected mice to test the hypothesis that NO is the suppressor factor in these cultures. However, the maximal reversal of suppression achieved with NMMA alone was less than 20% at physiological concentrations (up to 2 mM).

Inoculation of mice with 5×10^5 attenuated *Salmonella typhimurium* organisms induces high levels (30 to 50 μM per 2×10^6 cells) of NO as determined by nitrite accumulation using the Griess reaction. Several hypotheses that could account for the lack of efficacy of NMMA in reversing suppression of responses to ConA were considered. Among these were irreversible cytotoxicity to lymphocytes in mitogen-stimulated cultures. However, spleen cells from immune mice had the same viability as those taken from normal controls, as assessed by trypan blue exclusion both at the initiation of culture and at the time of cell harvest (reference 24 and data not shown). Furthermore, the ability to reverse suppression by combination treatments shows that cultures were viable. The possibility that other suppressor factors were present was considered. A combination of NMMA and indomethacin has been reported to reverse suppression in African trypanosomiasis (31). Although we had previously published our observation that prostaglandins are not the suppressor factors in the mitogen system after salmonella infection, as indomethacin was ineffective in reversing suppression (24), this experiment was repeated. Indomethacin at concentrations of up to 4 μg/ml was not found to restore responsiveness to ConA, and indomethacin was also not able to enhance the ability of NMMA to reverse suppression when the two compounds were used in combination (data not shown). A third explanation for the failure of NMMA

TABLE 4. Reversal of suppression of responses to mitogen by NMMA and anti-IFN-γ

No. of ConA-stimulated cells (10^5) and cell type		Treatment[a]	% Restoration of the normal ConA response[b]	Nitrite production (μM)[b]
Normal	Immune			
8				0 ± 0.4
8	2		1.0 ± 1.0	16.3 ± 0.1
8	2	NMMA	63.9 ± 6.9[c]	3.4 ± 0.8
8	2	α-IFN-γ	44.9 ± 5.9[c]	10.1 ± 1.2
8	2	NMMA plus α-IFN-γ	74.0 ± 3.0[c,d]	1.2 ± 0.9
8		NMMA plus α-IFN-γ		0.5 ± 0.5

[a] NMMA concentration = 1.25 mM. Anti-IFN-γ concentration = 200 ng/ml.
[b] Averages of data from two experiments ± standard errors.
[c] $P < 0.05$ versus value for no treatment.
[d] Not significant versus value for treatment with NMMA alone.

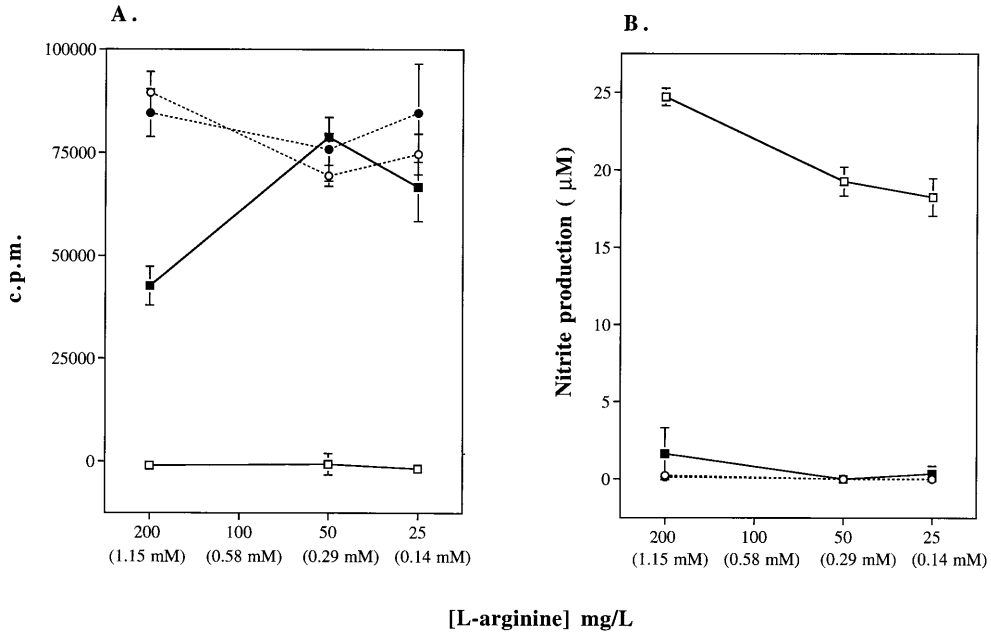

FIG. 3. Reversal of suppression of ConA-induced responses and NO production by NMMA (1.25 mM) combined with partial depletion of L-arginine in the medium. Cocultures of normal (8×10^5 per well) and immune (2×10^5 per well) spleen cells with (—■—) or without (—□—) added NMMA were placed in medium containing the standard concentration of L-arginine (200 μg/ml) or reduced concentrations of L-arginine with ConA. Responses of 8×10^5 normal spleen cells with (----●----) or without (----○----) NMMA are also shown. (A) [^{3}H]thymidine uptake measured 48 h later. (B) Nitrite production of the same wells measured 48 h after culturing. Data represent means ± SD of triplicate determinations from one of three similar experiments.

alone to reverse suppression was that ConA enhanced NO production, making reversal more difficult. This hypothesis was confirmed for cocultures. Further, it was shown that IFN-γ production was augmented along with nitrite production following ConA stimulation of immune spleen cells, as previously observed (1). A combination of IFN-γ and LPS is a major inducer of nitric oxide synthase in macrophages (1, 12, 25, 35). Since anti-IFN-γ partially inhibited nitrite production and also partially reversed suppression, it is tenable to conclude that the augmented NO induced by ConA prevented complete reversal of suppression by NMMA. In the PFC system, anti-IFN-γ had been 100% effective in reversing suppression of the PFC response (7). For mitogen-induced responses, anti-IFN-γ gave a less than 50% reversal unless it was combined with NMMA. There are several differences between the PFC assay and the mitogen system. First, in the PFC assay, the immune spleen cells are stimulated with antigen (sheep erythrocytes), not mitogen, which might be expected to induce less IFN-γ in the cultures. Second, exogenous sheep erythrocytes were added to PFC cultures during the incubation period. Hemoglobin is known to bind NO (3), thus reducing its effective concentration. In contrasting the mitogen and PFC assays, it can be concluded that the mitogen system maximizes NO levels in cultures and the PFC assay minimizes it. If the failure of NMMA to demonstrate high levels of efficacy in the mitogen system were due to excessive levels of NO, then reducing NO by other means should have augmented the reversal of suppression. The mitogen system was made less stringent in several ways. First, rather than using immune spleen cells alone, we cocultured immune spleen cells with normal cells. Second, NMMA was added to cultures to compete with arginine in the medium. Third, the arginine content of the medium was reduced to remove the substrate for NO production and to increase the probability that NMMA would bind to nitric oxide synthase. Under these conditions, a 100% reversal of suppression was obtained.

These results raise caution in interpreting the role of NO as the mediator of suppression in response to microbes by use of

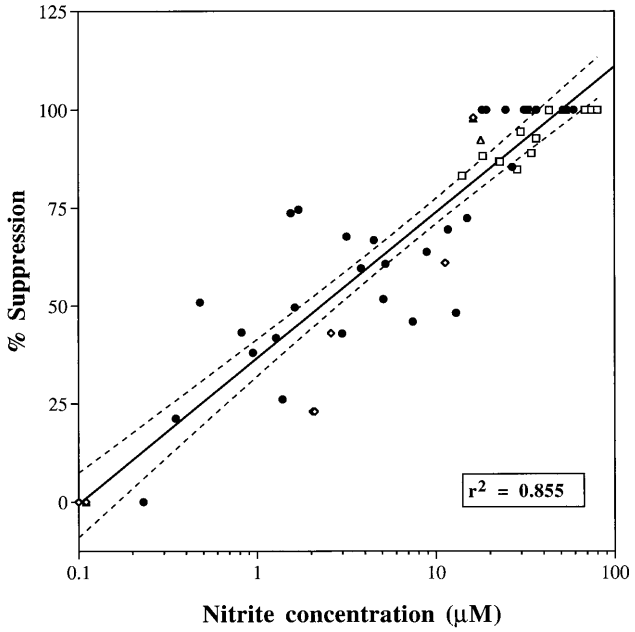

FIG. 4. Correlation between nitrite production and immunosuppression of ConA-induced responses. △, data from Fig. 1; ●, data from Fig. 2 and 3 and Table 2; □, data from Table 1; ◇, data from Table 4; ——, regression; -------, 95% confidence intervals.

NMMA. For example, incomplete reversal of suppression by NMMA in mitogen-stimulated spleen cells after exposure to *Trypanosoma brucei* or *Toxoplasma gondii* has led to the hypothesis that other suppressor factors might be involved (8, 34). Our results suggest that infection systems which generate high levels of NO may not be inhibitable with nontoxic concentrations of NMMA. When gram-negative organisms, such as salmonellae, are used, reversal of suppression may be most difficult because of the presence of LPS, which synergizes with IFN-γ in inducing macrophage nitric oxide synthase.

In this study of suppression of responses to mitogen, it was shown that unfractionated spleen cells and adherent cells enriched for mature macrophages were both suppressive in cocultures and produced comparable levels of nitrite. The adherent cell fraction was not more active than the unfractionated cells. One explanation for this observation comes from work in the PFC system, in which immature macrophages were shown to be involved in immunosuppression, as was shown for mature macrophages (4, 5). Immature macrophages do not produce nitrite unless they are treated with IFN-γ (21). Thus, the comparable suppressive ability of unfractionated cultures and adherent fractions can be explained by the presence of significant numbers of immature macrophages in the immune spleens inoculated 7 days earlier, which were induced to make NO by the IFN-γ stimulated by ConA treatment. Anti-IFN-γ may be only partially effective, because it blocks induction of inducible nitric oxide synthase in the immature macrophages but not in the mature ones. Another consideration is the complex relationship between IFN-γ levels and NO levels, as described for T-cell clones (33, 37), which showed that NMMA augmented IFN-γ levels by blocking the inhibitory effect of NO on T-cell function. Thus, reducing NO levels with NMMA could have increased IFN-γ levels, establishing a positive feedback loop for NO that could have allowed greater inducible nitric oxide synthase induction in precursor macrophages.

A cursory examination of the relationship between nitrite levels and suppression does not show an easily observable correlation. In many cases, addition of NMMA or anti-IFN-γ or decreasing the arginine concentration caused significant percent reductions in nitrite levels but suppression was still appreciable. These apparent inconsistencies can be reconciled by examining individual values of percent suppression against the logarithm of nitrite in a standard linear regression. The trend, indicated by the value of r^2, is indicative of a good fit and a significant dose-effect relationship ($P < 0.05$), as seen by the confidence interval of the slope. A consequence of this relationship is that ConA-stimulated responses can be completely suppressed when nitrite levels are at 15 μM or higher. When nitrite concentrations are low (1 to 10 μM), a wide range of suppression (25 to 75%) is observed. An important observation is the variability in suppression observed when nitrite levels are below 5 μM. The confidence intervals show that for log-linear relationships, there is increased variation as one moves away from the mean. The Griess reaction is thus least sensitive and most variable at nitrite levels below 5 μM.

The results presented in this paper show that nitric oxide is the inhibitor of responses to ConA in spleen cells of mice immunized with attenuated *Salmonella typhimurium*. The experiments also show that using the capacity of NMMA to reverse suppression as the criterion for involvement of NO as a suppressor factor can yield misleading results, especially in ConA-driven mitogen systems. NMMA may be unable to provide complete reversal of suppression because of increased IFN-γ production triggered by ConA that increases NO levels. A major finding in these studies is the log-linear relationship between nitrite concentrations and immunosuppression, such that small amounts of NO, which are not reliably measured by the Griess reaction, may be present and immunosuppressive in immune spleen cell cultures.

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