The production of nitric oxide (NO) was measured in cultures of spleen cells stimulated by lipopolysaccharide (LPS), IL-2 or LPS + IL-2. We observed that NO synthesis is increased by IFN-γ but inhibited by IFN-α/β. This is not the case when IL-2 is present in the cultures, since interferons play a minor role in the regulation of the NO production. When IL-2 and LPS were associated in the cultures, the IFN-α/β role seems more important than that of IFN-γ. PGE2 inhibits NO production in LPS supplemented cultures but has a slight effect in the presence of IL-2 and no effect with IL-2 + LPS. 3-IsoButyl-1-methylxanthine (IBMX), an inhibitor of phosphodiesterases, induces a decrease of IFN production. In the presence of H-7, an inhibitor of protein kinase C (PKC), NO production is reduced when the cultures are supplemented by LPS or IL-2 but not when IL-2 and LPS are both added. H-7 also reduced IFN production. In the presence of N6-monomethyl-L-arginine (N-MMA), an inhibitor of NO synthesis, IFN production was increased, with no change in the cytotoxic activity. Hence, interferons regulate NO production by mouse spleen cells and, in return, NO modulates the generation of IFN.

**Key words:** Cytotoxicity, Interleukin-2, Lipopolysaccharide, Nitric oxide

### Introduction

Nitric oxide (NO) is important in many biological functions. It is generated from L-arginine by the enzyme NO synthase (NOS). Peritoneal macrophages of mice are the best-characterized source of NO. LPS and interferon-γ (IFN-γ) constitute the major stimulating factors of NO production by increasing cellular concentrations of NOS. Many functions of LPS have been reported to be mediated by prostaglandin production, which in turn acts by increasing cAMP. The role of PGE2 on NO production is unclear. PGE2 did not affect NO production nor did it inhibit NO production. Other work shows that NO activates cyclooxygenase enzymes and leads to a marked increase in PGE2 production.

Intracellular mechanisms induced by LPS implicate protein kinase C (PKC) and cAMP dependent protein kinase A. LPS causes the translocation of protein kinase C from cytosol to the membrane. PKC has been involved in the induction of NO synthase activity in macrophages by IFN-γ. Another report suggested rather the role of protein kinase A.

The studies on NO production and NO regulation have been performed essentially with purified macrophage populations. We have shown in previous work that LAK activity induced by IL-2 was reduced by LPS and that the decrease was related to the increase of IFN-α/β production. On the other hand, NO was implicated in LAK activity in rodents. Thus the aim of this study was to evaluate NO production induced by splenic macrophage cultures with other splenic cell populations and their cytokines after stimulation by LPS and IL-2, and to assess if there is a relationship between the generation of cytotoxic activity and NO production.

We have investigated whether NO, measured as nitrite (NO2-), is detected in supernatants of spleen cells stimulated for 3 days in the presence of LPS, IL-2 or LPS + IL-2, and the role played by interferons induced by IL-2 and LPS on NO generation. We have also examined whether NO production was regulated by PGE2 and by LPS-induced PKC activation. Finally we have investigated whether NO plays a role in the generation of cytotoxic activity.

### Materials and Methods

**Mice:** Inbred C3H mice, 6–8 weeks old, were used throughout the experiments.
Reagents: Lipopolysaccharide from Escherichia coli 055:B5 was purchased from Difco. Anti-IFN-α/β serum (1:320000) was a generous gift from Dr. Ion Gresser (IRCS, Villejuif, France). Antimurine IFN-γ antibodies were purchased from Genzyme (Boston, MA). rIL-2 was purchased from Cetus Corporation (Emeryville, CA) and PGE2, IBMX, N-MMA and H-7 from Sigma (St Louis, MO).

Cell lines: YAC-1 and I 929 cells were maintained in vitro. YAC-1 is a Moloney virus-induced lymphoma of A/Sn origin. I 929 is a fibroblast cell line derived from an adult C3H mouse.

Preparation of spleen cells: Spleen cells were harvested and suspended in phosphate-buffered saline solution (PBS). Erythrocytes were removed by osmotic shock and the final cell suspension was resuspended in RPMI 1640 culture medium supplemented with 5% foetal calf serum, L-glutamine (2 mM), Hepes (10 mM), penicillin (100 U/ml), streptomycin sulfate (100 μg/ml), and 2-mercaptoethanol (0.05 mM), which constitutes complete medium.

Measurement of NO₂⁻ production: NO was estimated by measuring the formation of NO₂⁻ (the stable oxidative end product of NO) which serves as a quantitative index of macrophage activation. NO₂⁻ levels in culture fluids were estimated by using the Griess reagent.14 Briefly, 100 μl culture fluid was incubated with an equal volume of 1% sulfanilamide and 1% N-1-naphthylethylenediamine dihydrochloride in 2.5% H₃PO₄ (Sigma) at room temperature for 5 min. NO₂⁻ was quantified by using spectrophotometry to measure the OD at 570 nm, with NaN₂O₂ as a standard.

Inhibition of NO₂⁻ synthesis: The specific inhibitor of NO synthase, N⁵-monomethyl-L-arginine (N⁵-NMA) was added at a concentration of 50 μM to the spleen cell culture.

Cytotoxicity assay: Effector spleen cells were assayed using [³⁵]Cr-Na₂CrO₄-labelled (Amersham, UK) target cells. Cells from the YAC line were used as targets. Cytotoxic activity was measured for different spleen/target cell ratios in a final volume of 200 μl. After 4 h incubation at 37°C, 100 μl supernatants were removed from each well for counting. Specific lysis was calculated as:

\[
\% \text{ lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100
\]

Spontaneous release was obtained by incubating targets alone and total release was determined by collecting 100 μl from cells in which the pellet had been resuspended. All assays were performed in triplicate. The S.E.M. of the mean was within 5–10% and was not represented. Results of cytotoxicity assays were expressed either in percent of lysis for different effector/target cell ratios or in lytic units (LU)/10⁷ cells. LU was calculated from linear regression curves plotted from the various E/T ratios. One LU was defined as the number of effector cells required to lyse 20% of the target cells.

IFN assay: The presence of IFN was assayed by anti-vesicular stomatitis virus (VSV) activity. I 929 cells were seeded in 96-well (3 x 10⁴/well) flat-microtire plates. Serial dilutions of supernatants were added. After 18 h at 37°C, the cells were infected with VSV at high multiplicity of infection. The plates were scored after 24 h. The antiviral effect was estimated by measuring the incorporation of red neutral dye into living cells according to the method described by Hudson and Hay5 and using a Titertek multispec tern spectrophotometer. The IFN titre of a supernatant was estimated as the reciprocal of the dilution inhibiting 50% of the cytopathic effect.

Results

Release of NO in spleen cells cultured in the presence of LPS, IL-2 or LPS + IL-2: Initially we tried to determine whether under our experimental conditions, splenocytes could be stimulated by IL-2, LPS or both, to produce NO. For that purpose spleen cells were cultured for 3 days in the presence of increasing concentrations of the inducers used either alone or associated. NO₂⁻ was assessed in supernatants by the Griess procedure. The results of one out of three similar experiments are shown in Fig. 1. We observed that the response to LPS was induced with low concentrations of reagent (1 ng/ml), increased and reached a plateau for a range of concentrations from 100 to 10000 ng/ml. NO induction was possible with 100 U/ml of IL-2, and was even greater when LPS and IL-2 were present in the culture. One should underline that even if IL-2 alone at 10 U/ml did not induce NO synthesis it can act synergistically with LPS.

In a previous report12 it was demonstrated that IL-2-induced LAK activity was diminished by LPS (1 μg/ml). As the aim of this work was to investigate whether NO production was involved in that decrease, the following experiments were performed with 1000 U/ml of IL-2 and 1 μg/ml of LPS.
Implication of interferons in the production of nitric oxide: IL-2 is an inducer of IFN-γ. LPS is also an inducer of IFN-α/β and the association of IL-2 with LPS induces a synergistic increase in the production of IFN-α/β and IFN-γ. Moreover, IFN-α/β is implicated in the change in cytotoxic activity induced by LPS on IL-2-stimulated spleen cells.

We have studied the effects of IFN-γ or IFN-α/β on NO production induced by LPS after 72 h of culture. For this purpose 100 U/ml of IFN-γ or IFN-α/β were added to spleen cells cultured in the presence of LPS at 1 μg/ml. Table 1 summarizes four experiments. It was observed that IFN-γ induced an increase of NO₂⁻ (+128%) while IFN-α/β reduced NO₂⁻ production (−34%).

We then determined if the interferons induced by LPS, IL-2 or a mixture of both played a role in NO production. For that, the culture was incubated in the presence of anti-IFN-γ antibodies or anti-IFN-α/β anti-serum, and NO₂⁻ was measured after 3 days. Table 2 presents the results of three experiments. We observed that for LPS cultures the anti-IFN-γ antibodies reduced NO production. This effect of anti-IFN-γ antibodies was diminished in cultures stimulated by IL-2 or IL-2 + LPS. The presence of anti-IFN-α/β serum increased NO production by 40% for LPS and IL-2 + LPS cultures, with smaller increases in IL-2 cultures.

The role of cAMP on NO production by spleen cells cultured in the presence of IL-2 or IL-2 + LPS, in relation to IFN production: LPS-stimulated macrophages produce PGE₂ which induces a transient increase of cAMP production. cAMP is degraded by phosphodiesterases. For testing the effects of endogenous PGE₂ stimulated by LPS, we added IBMX, an inhibitor of phosphodiesterase, to the cultures and therefore promoted the accumulation of cAMP in the culture medium. NO production was measured after 3 days. Results were presented as the percent variation of NO between the IBMX group and the untreated group. The percent variation of five experiments is shown in Table 3. The produc-
NO, absence of implication in cytotoxic activity induced by LPS

Table 4. Effect of H-7 presence on the NO produced by spleen cells cultured for 72 h with LPS, IL-2 or LPS + IL-2

| Experiment | LPS | IL-2 | LPS + IL-2 |
|------------|-----|------|-----------|
| 1          | 65  | 70   | 6         |
| 2          | 75  | 62   | -13       |
| 3          | 64  | 54   | +9        |
| 4          | 68  | 62   | +4        |

Mean ± S.E.M. - 68.0 ± 2.5, 62.0 ± 3.3, 1.5 ± 4.9

*% variation A - B/B x 100 in which A is NO measured in H-7 culture (20 µM) and B is NO measured in culture without H-7.

Role of NO in the generation of LAK cells and IFN production: We have found NO production by spleen cells cultured with LPS, IL-2 or IL-2 + LPS. We have examined if NO is involved in the generation of cytotoxic cells of the LAK type and in IFN production. In these experiments NO, which is derived from a terminal guanidino nitrogen from arginine, is blocked by the L-arginine analogue N^6-monomethyl arginine (N-MMA). Spleen cells were cultured for 3 days with LPS (1 µg/ml), IL-2 (1000 U/ml) or IL-2 + LPS in presence or absence of N-MMA (50 µM).

NO and IFN levels were measured in the supernatants and the cells were collected and tested for their cytotoxic activity against YAC cells for different effector-target cell ratios (10:1 to 0.15:1). Cytotoxic activity was expressed in lytic units. Results of four experiments are reported in Table 5.

We observed that the production of NO falls in the presence of N-MMA, while IFN production was always increased. There was a non-significant trend for cytotoxic activity to increase in the presence of N-MMA.

Discussion

We have examined NO production in murine spleen cell cultures stimulated with either LPS, IL-
Table 5. The effects of N-MMA presence on NO\textsuperscript{2} and IFN production and generation of cytotoxic activity in spleen cells cultured for 3 days with LPS (1 \mu g/ml), IL-2 (1000 U/ml) or IL-2 + LPS

| Experiment | Substance(s) added | NO\textsuperscript{2} (nmol/ml) | IFN (U/ml) | Cytotoxic activity (LU) |
|------------|-------------------|-------------------------------|------------|------------------------|
|            |                   | Without N-MMA | With N-MMA | Without N-MMA | With N-MMA | Without N-MMA | With N-MMA | Without N-MMA | With N-MMA |
| 1          | LPS               | 10               | 2          | 12            | 14          | 51            | 60         |
|            | IL-2              | 13               | 6          | 18            | 29          | 500           | 667        |
|            | IL-2 + LPS        | 23               | 5          | 139           | 192         | 138           | 168        |
| 2          | LPS               | 20               | 3          | 11            | 2           | 72            | 76         |
|            | IL-2              | 20               | 3          | 11            | 322         | 625           | 1180       |
|            | IL-2 + LPS        | 25               | 5          | 125           | 322         | 312           | 480        |
| 3          | LPS               | 11               | 1          | 3             | 5           | ND            | ND         |
|            | IL-2              | 9                | 1          | 21            | 34          | 1049          | 1250       |
|            | IL-2 + LPS        | 23               | 2          | 153           | 256         | 232           | 312        |
| 4          | LPS               | 15               | 1          | ND            | ND          | ND            | ND         |
|            | IL-2              | 13               | 1          | 13            | 15          | 1000          | 1050       |
|            | IL-2 + LPS        | 23               | 1          | 100           | 1421        | 285           | 222        |

\( ^a \)LU lytic units calculated at 20% cytotoxicity/10\textsuperscript{7} cells.
\( ^b \)N-MMA 50 \mu M.

2 or LPS + IL-2 for 3 days. We observed similar NO production in cultures containing IL-2 and in those containing LPS. It is known that only LPS and IFN-\gamma are stimulating factors of NO production by macrophages.\textsuperscript{12} The NO induced in the IL-2-stimulated spleen cell cultures was probably attributable to IFN-\gamma induced by IL-2.\textsuperscript{16} The production of NO was significantly increased when LPS and IL-2 were combined in the cultures.

LPS induced low levels of IFN. The association of IL-2 with LPS induced a synergy in IFN production.\textsuperscript{12,18} Similarly to the work of Ding \textit{et al.}\textsuperscript{2} in macrophage cultures, we observed that IFN-\gamma increases the NO induced by LPS in spleen cell cultures but different effects were observed with IFN-\alpha/\beta addition. In the latter, we observed a significant reduction of NO production, whereas Ding \textit{et al.} observed that IFN-\alpha or IFN-\beta alone were not inducers of NO, but their association with LPS increased NO production although to a lesser degree than IFN-\gamma.

This opposing effect that we have observed, depending upon the type of IFN added to LPS, was confirmed when spleen cells were cultured in the presence of either anti-IFN-\gamma antibodies or anti-IFN-\alpha/\beta serum. When the cultures were made in the presence of anti-IFN-\gamma antibodies, we observed (Table 2) a reduction of NO induced by LPS and to a lesser degree by LPS + IL-2, but there was no effect on IL-2 cultures. In the presence of anti-IFN-\alpha/\beta serum a similar increase of NO was observed in LPS or LPS + IL-2 culture and a lower increase for IL-2 cultures. This result differed from that of Riches and Underwood\textsuperscript{20} who found that the anti-IFN-\alpha/\beta presence inhibited the NO release by macrophages stimulated by IFN-\gamma and poly IC.

Thus, IFN-\alpha/\beta has immunomodulatory properties which differ from those of IFN-\gamma for the modulation of NO production. Different effects of these two types of IFN have been described for induction of IL-1,\textsuperscript{21} induction of cytotoxic activity\textsuperscript{12} and ConA and IL-2-induced proliferation of spleen T-cells.\textsuperscript{22}

If IFN-\gamma is an inducer of NO, it must be emphasized that NO is an inhibitor of IFN production. Indeed when the cultures were made in presence of an inhibitor of NO, IFN production was increased.

We observe that independent of the stimulating agent, the reduction of NO by N-MMA was followed by a small increase in cytotoxic activity in nine of ten cytotoxic assays (Table 5). In contrast with our results, Juretic \textit{et al.}\textsuperscript{13} reported that reduction of NO production induced a decrease of LAK activity in rodent spleen cells. At present, we do not have an explanation for this discrepancy.

The increase of IFN production and the weak effect on cytotoxic activity in cultures made in the presence of an inhibitor of NO may be secondary to a reduction of PGE\textsubscript{2}. Indeed Salvemini \textit{et al.}\textsuperscript{8} have shown that PGE\textsubscript{2} production was associated with NO production which activates cyclooxygenase enzymes. So we hypothesize that the fall of NO induced a reduction of PGE\textsubscript{2}, which explains the increase of IFN. PGE\textsubscript{2} is known to be an inhibitor of IFN production\textsuperscript{23} and cytotoxic activity generation.\textsuperscript{24}

In our system LPS induced PGE\textsubscript{2} because (a) IFN production and cytotoxic activity were increased in the presence of indomethacin, which is an inhibitor of cyclooxygenase pathway;\textsuperscript{12} and (b) IBMX which produces a
cAMP accumulation inhibits NO production (Table 4). On the other hand exogenous PGE$_{2}$ inhibits NO induced by LPS (Fig. 2). Unexpectedly, the presence of indomethacin which should provoke an increase of NO synthesis by inhibition of PGE$_{2}$ production, caused a slight decrease of NO production. A similar effect was observed by Schleifer and Mansfield. So cAMP accumulation in the presence of IBMX was not necessarily related to a PGE$_{2}$ effect. This is in agreement with the data of Piguet-Pellorce and D$_{y}$ who reported that the increase in histamine release by bone marrow cells stimulated by LPS plus GM-CSF, was related to an increase of cAMP, which is not related to PGE$_{2}$ synthesis. In contrast, PGE$_{2}$ can increase NO production by LPS-stimulated Kupffer cells. In IL-2 or IL-2 + LPS cultures, the presence of IBMX reduced NO production, although the inhibition was less than in LPS culture; however, IFN production was drastically reduced. On the other hand PGE$_{2}$ induced a slight but significant reduction of NO in IL-2 cultures, but we did not observe a PGE$_{2}$ effect in LPS + IL-2 cultures. This suggests that IL-2 or IL-2 + LPS induce the release of factors which counterbalance the inhibitory effect of PGE$_{2}$. Moreover IL-2 has been reported to partly reverse the inhibitory effect of PGE$_{2}$.

The signal transduction pathway by which IFN-γ induces NO production remains to be established. PKC is involved in the induction of nitric oxide synthase by IFN-γ, protein kinase C activity is increased by IFN-γ but not by IFN-α/β or LPS. Work by Fujihara et al. showed that PKC plays an important role in the LPS-triggered signal transduction pathway. However, other different protein kinases such as PKA via PGE$_{2}$ induction may be involved. There is also a link between PKA-mediated signalling and nitric oxide synthase. Using H-7, an inhibitor of PKC, we observed a similar reduction of NO that is induced by LPS or IL-2. No effect was observed when the cells were cultured in the presence of IL-2 + LPS, but the presence of H-7 induced a large reduction (97%) of IFN induced by LPS + IL-2. Gessani et al. showed that treatment of macrophages by staurosporine, an inhibitor of PKC, stimulated by IFN-γ inhibits IFN secretion.

There is a relationship between intracellular cAMP concentration and PKC activation. H-7 was an inhibitor of PKC and also of PKA. One might hypothesize that the inhibition of NO induced by H-7 may correspond to the PKA inhibition of LPS, and the PKC inhibition of IL-2. H-7 did not affect NO production in LPS + IL-2 culture but reduced IFN production. It must be emphasized that inhibition of PKC may involve the reduction of the inhibitors of NO production such as TGF-β and/or IL-4. On the other hand, as we have shown, it may be that IFN-α/β plays an inhibitory role in NO production, and the fall of IFN-α/β might explain the maintenance of NO levels.

We underline that two metabolic inhibitors, i.e., IBMX and H-7, which did not affect the NO level, reduced IFN production in IL-2 + LPS cultures, whereas NMMMA, which inhibited NO production, increased IFN levels.

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