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Anti-MHV3 state induced by IFN gamma in macrophages is not related to arginine metabolism

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Summary. In contrast to BALB/c mouse macrophages, the A/J macrophages after activation by interferon gamma (IFN gamma) develop an anti-MHV3 effect which correlates with the resistance to virus infection. To understand the cellular basis of this antiviral effect, we studied the possible involvement of arginine metabolism through nitric oxide (NO) and arginase induction, since these metabolic pathways have been described as implicated in antiviral activities of macrophages. The studies were performed by activating macrophages with inducers of NO (IFN gamma) and arginase (IL4 IL10). NO synthase (iNOS) and arginase inhibitors (N-methyl-arginine, NMA, and hydroxyarginine, OH-ARG) were used. The results show that in both macrophage populations, no spontaneous synthesis of NO occurred and the MHV3 enhanced the NO release induced by IFN gamma. After activation with IFN gamma, BALB/c macrophages released higher amounts of NO than the A/J macrophages. The inhibition of IFN gamma-induced NO-synthesis with NMA or with arginine free medium did not affect the virus replication. In BALB/c macrophages, IL4 or IL10, induced higher amounts of arginase than in A/J macrophages. In both macrophage populations the MHV3 infection had no influence on the arginase synthesized, and the inhibition of the arginase with OH-ARG had no influence on the virus growth. The level of MHV3 replication or inhibition was also not influenced when we used macrophages from knock-out mice for the iNOS gene, and as a consequence were unable of synthesizing NO. These data indicate that NO and arginase do not participate in the anti-MHV3 state induced by IFN gamma in macrophages.

Introduction

The Mouse Hepatitis Virus 3 (MHV3) is used as a model of viral infection in which resistance is dependent on the genetic background of the mouse strain

[8, 17, 29, 36]. The differential magnitude of the antiviral state induced by interferon gamma (IFN gamma) in macrophages from resistant (A/J) and susceptible (BALB/c) mouse strains were shown to be specific for MHV3, correlating with the host resistance to the experimental viral infection [25]. Our previous studies have shown that resistance to MHV3 infection in mouse populations can be a consequence of a T-cell dependent mechanism, in which the production of IFN gamma and the sensitivity of macrophages to IFN gamma play an essential role [19, 20, 25, 36, 37].

In the attempt to elucidate the cellular basis of the macrophage expression of antiviral state in response to IFN gamma, we have shown that the release of TNF alpha, IL-1, O_2^- and H_2O_2 by A/J and BALB/c macrophages upon IFN gamma activation and/or MHV3 infection seems not to account for the antiviral state exerted by A/J macrophages and may contribute to the susceptibility of BALB/c mice by playing a pathological role [21, 35].

In macrophages, NO synthase (iNOS) and arginase are inducible enzymes that act on a common substract, arginine. As a consequence, arginine is oxidized by iNOS producing nitric oxide (NO) and citrulline or is hydrolyzed by arginase producing ornithine and urea [12]. We have recently shown that a competition of both enzymes for their substrate takes place in mouse bone marrow-derived macrophages and that the iNOS and arginase appear to define two alternate functional states of macrophages, induced by, respectively, TH1 (IFN gamma) and TH2 cytokines (IL4 and IL10) [26].

NO has been considered an important effector molecule with protective or harmful effects on the organism and directly involved in antiviral activities [6, 10, 15, 33]. The inhibition of ectromelia, vaccinia and herpes simplex 1 viruses in IFN gamma activated mouse macrophages correlated with the cell production of NO [11, 13]. It has been shown the iNOS induced in macrophages of mice infected with Coxsackie B3 virus and a higher mortality of these infected mice when fed iNOS inhibitors [18]. A direct antiviral effect of NO with inhibition of vaccinia virus replication at the level of DNA synthesis has also been shown [24]. Recent findings indicate that NO prevents Epstein-Barr virus replication by inhibiting viral DNA amplification and by blocking activation of the latent viral genome [14]. Published data suggest that signaling requirement of NF-kB activation and NO production in Newcastle disease virus-treated macrophages are similar [34]. On the other hand, there are indications that NO may not be essential for the virus clearance, since treatment of vaccinia virus-infected mice with the iNOS inhibitor did not alter the course of infection [30]. In a recent publication, Kreil and Eibl [16] showed that NO has no antiviral activity against tick-borne encephalitis virus infection in murine macrophages and can even contribute to pathogenesis in experimental infection.

In contrast, the arginase induction in macrophages as well as its consequences has been poorly studied [4, 5, 26]. Early reports implicate the arginase in antiviral activities in macrophages, tumor cell cytotoxicity and immunosuppression during mixed leukocyte culture [31, 32, 39].

In view of the differential role of macrophages from resistant or susceptible mice, in restricting the replication of MHV3 upon activation by IFN gamma, we have drawn attention to the arginine metabolism as a possible biochemical basis for explaining the IFN gamma induced anti-MHV3 effect in macrophages from resistant and susceptible mice.

Materials and methods

Mice, macrophages and virus

A/J and BALB/c mice were obtained from the mouse colony of the Max-Planck Institut for Immunobiology, Freiburg, Germany. C57BL/6×129Sv knockout mice deficient in inducible iNOS (iNOS−/−) and its wild type counterparts (iNOS+/+) were kindly supplied by J. D. MacMicking and C. Nathan [22]. They were kept under conventional conditions and used at 4 to 8-week-old. Care of animals followed the appropriated guidelines.

Bone marrow-derived macrophages of mice were obtained from bone marrow cells collected from the femurs by flushing the cavities with Dulbecco's modified MEM (DMEM)(Biochrom, Berlin) supplemented with 100 units (U)/ml of penicillin (Sigma) and 100 µg/ml stretomycin (Sigma). Cells were cultured in non-toxic Teflon bags (fluorinated ethylene propylene, Biofolie 25; Heraeus) as described [1, 27]. The Teflon foils were folded, sealed with a diathermal sealing apparatus (Polystar 100 B and Polystar 401 M-RPA; Riesche-Herfurth) to give rectangular bags and subjected to gas sterilization. Cells (5×10^4/ml) in DMEM with 10% fetal calf serum (FCS)(Biochrom, Berlin), 5% horse serum (Gibco), 15% L929 supernatant as a source of colony-stimulating factor, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% sodium pyruvate (Gibco) and 0.5% 2-mercaptot-hanol (Sigma) were incubated for 12 days at 37 °C in the presence of 10% CO_2. Cells were detached by repeated careful stretching of the Teflon bags, washed once with medium and used in the experiments. MHV3 was cloned by limiting dilution, one plaque was selected and amplified on L929 cells to serve as the inoculum for future stocks [23] to limit spontaneous mutations. The stocks were always titrated by plaque assay on L929 cells as previously described [28]. Aliquots containing 2×10^5 plaque forming units per milliliter (PFU/ml) were stored at −80 °C and used in all experiments.

Macrophage treatment and/or infection

Bone marrow-derived macrophages from mice, cultured in DMEM containing 10% FCS at a concentration of 10^5 cells/well in 96-well plates, were treated for the indicated time with 100 U/ml of recombinant IFN gamma (Dr. Adolf, Ernst-Boehringer-Institut, Vienna) or 10 U/ml of recombinant IL10 (Pepro-Tech, London, UK) or 10 U/ml of recombinant IL4 (R-D Systems, Abingdon, UK) or 0.5 mM of N-methyl-arginine (NMA) (Alexis Gruenberg, Germany) or 0.5 mM of hydroxyarginine (OH-ARG) (Alexis Gruenberg, Germany). The macrophages were alternatively cultivated in arginine free medium (-ARG) (Biochrom, Berlin). In all the experiments the macrophages were infected with 0.1 moi of MHV3 and the virus growth measured in the culture supernatants 24 h after infection [28].

Measurement of arginase activity and NO production in macrophages

Arginase activity was measured in macrophage lysates as previously described [3]. Briefly, 10^6 cells were lysed with 0.5 ml 0.1% Triton X100 and after 30 min 0.5 ml of a buffer

containing 25 mM Tris-HCl and 5 mM Mn Cl_2, pH 7.4 was added. The enzyme was then activated by heating for 10 min at 56 °C. Arginine hydrolysis was carried out by incubating 25 µl of the activated lysate with 25 µl of 0.5 M arginine, pH 9.7 at 37 °C for 60 min. The reaction was stopped with 400 µl of an acidic mixture (H_2SO_4, H_3PO_4 and H_20, 1:3:7 v/v). The urea was measured at 540 nm after addition of 25 µl of 9% alpha-isonitrosopropiophenone (dissolved in 100% ethanol) and then heating at 100 °C for 45 min. One unit of enzyme activity is defined as the amount of enzyme which catalyses the formation of 1 µmol urea/min. NO was measured as nitrite using the Griess reagent. Culture supernatant (100 µl) was mixed with 100 µl of 1% sulfanilamide, 0.1% naphtylenediamine dihydrochloride and 2.5% H_3PO_4. Absorbance was measured at 540 nm in a ELISA reader (Biochrom, Berlin). Results of all experiments are the mean values with standard deviations of triplicate cultures.

Results

No release in A/J and BALB/c mouse macrophage cultures

As shown in Fig. 1, no spontaneous synthesis or NO occurred in both mouse macrophage populations. Following the IFN gamma activation, NO release occurred and higher amounts were observed in BALB/c mouse macrophage cultures. When the cultures were performed in the presence of arginine free

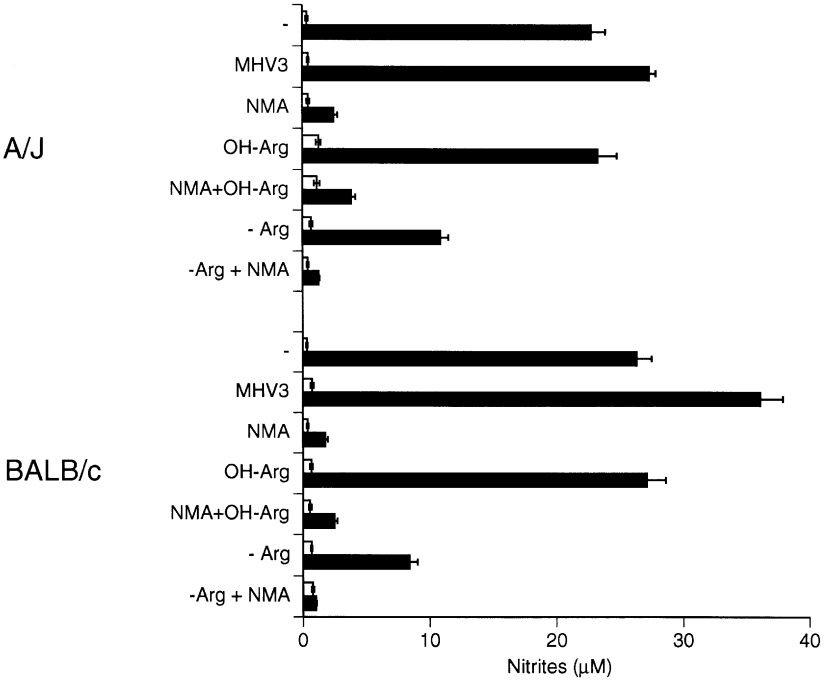

Fig. 1. Induction of NO in macrophage cultures of A/J and BALB/c mice. Cell cultures were infected with 0.1 moi of MHV3 or performed in absence of arginine (-Arg) and/or presence of 0.5 mM of N-methyl-arginine (NMA) and/or 0.5 mM hydroxyarginine (OH-Arg). The cultures were activated for 18 h with 100 U/ml of IFN gamma and the nitrites concentration measured in the supernatants is expressed in uM with standard deviations. IFN gamma activated (closed bars) and control cultures (open bars) are shown

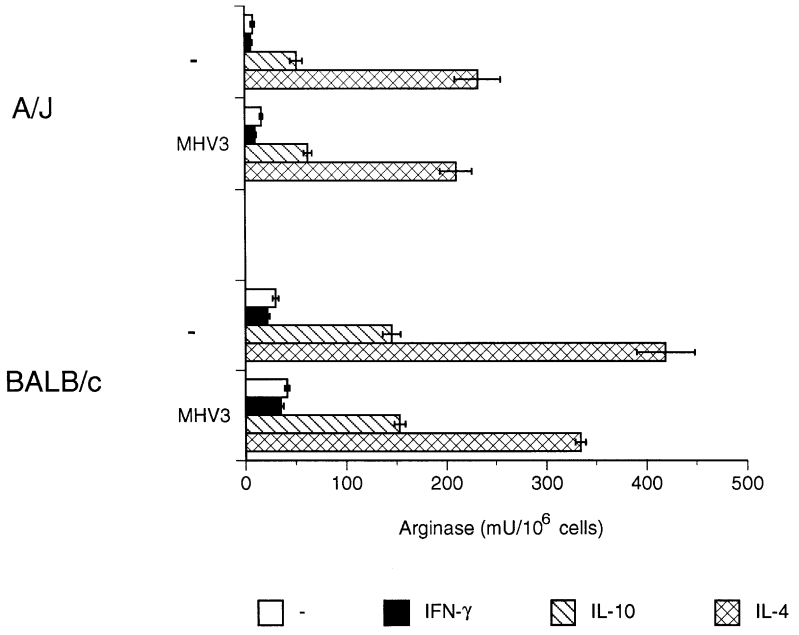

Fig. 2. Expression of arginase in macrophage cultures of A/J and BALB/c mice. Cell cultures were activated for 18 h with 100 U/ml of IFN gamma or 10 U/ml of IL4 or IL10. The arginase concentrations measured in the supernatants 18 h later are expressed in mU/10^6 cells with standard deviations

medium (-ARG) or N-methyl-arginine (NMA) or both, a drastic decrease in the synthesis of NO was observed. The data shown also that the MHV3 infection was capable of further enhance the IFN gamma triggered-NO release from both mouse macrophage populations.

Arginase induction in A/J and BALB/c mouse macrophage cultures

The data presented in Fig. 2, confirming our previous data [26], indicate that the background levels of arginase were higher in BALB/c mouse macrophages than in A/J ones. Also in agreement with our previous data [26], in both macrophage populations, the IFN gamma treatment had a small suppressive effect on the background levels of arginase expression. The MHV3 infection did not influenced significantly the arginase expression, the lower levels observed in MHV3-infected BALB/c mouse macrophages being due to the cell destruction taking place during the virus infection. On the other hand, the IL4 or IL10 were capable of inducing arginase expression in both macrophage populations which was higher in A/J mouse macrophages.

MHV3 replication in activated/inhibited A/J and BALB/c mouse macrophage

In contrast to BALB/c mouse macrophages, the A/J ones, after IFN gamma activation, are able to partially inhibit the virus growth. This effect, which can

Table 1. MHV3 growth, NO synthesis and arginase expression in macrophages from iNOS−/− and iNOS+/+ mice activated or not with IL4 and IFN gamma[a]

Mo	MHV3 (PFU/ml)		Nitrites (uM)		Arginase (mU×10^6 cells)	
	–	IL4/IFN g	–	IL4/IFN g	–	IL4/IFN g
iNOS−/−	1(0.2)×10^4	6.5(0.1)×10^2	0.9(0.1)	0.7(0.2)	3.1(0.8)	204.4(1.8)
iNOS+/+	3(0.1)×10^4	1.2(0.3)×10^3	0.7(0.1)	34.8(1.6)	6.4(0.8)	246.1(1.7)

[a] Cultured macrophages isolated from mice were treated for 24 h with 10 U/ml of IL4, for 18 h with 100 U/ml of IFN gamma and then infected with 0.1 moi of MHV3. MHV3 titer (PFU/ml) or nitrites (uM) and arginase (mU ×10^6 cells) release were measured in supernatants of macrophage cultures 24 h after virus infection. The results expressed are the average of three different experiments with standard deviations

be induced by IFN gamma activation only in A/J cells, could not be reversed by the macrophage treatment with inhibitors of NO or arginase synthesis (-ARG, NMA or OH-Arg). Also the MHV3 replication in BALB/c macrophages, regardless of the IFN gamma activation, was not influenced by the treatment with the NO or arginase inhibitors (data not shown).

NO or arginase activity and MHV3 replication in iNOS−/− and iNOS+/+ mouse macrophage cultures

In Table 1 we show that in both iNOS−/− and iNOS+/+ mouse macrophages, the MHV3 could replicate to the same level and were similarly inhibited following IFN gamma/IL4 activation. As expected, in contrast to the macrophages from the wild type (iNOS+/+), the iNOS−/− were not capable of releasing NO following activation. Both macrophage populations showed comparable expression of arginase following activation.

Discussion

The mouse hepatitis virus strains of coronavirus are responsible for endemically occurring enteritis in most mouse colonies [7]. It has been speculated that the macrophages play a central role in determining the resistance to experimental infection of mice with MHV3 [2, 38]. The immune response, the virus replication, the antiviral state induced by interferon and the expression of a monokine with procoagulant activity in macrophages have been implicated in the resistance/susceptibility of mouse strains to MHV3 infection [8, 9, 17, 19, 20, 29, 36, 37]. We have shown that the MHV3-susceptible BALB/c mice have macrophages where the virus can grow to high titers and are not restricted when they are activated with IFN gamma. In contrast, the MHV3-resistant A/J mice have macrophages that respond to IFN gamma activation, partially restricting the virus growth [19, 20, 25, 36, 37]. The cellular/molecular basis of this

phenomena is not yet known, and this study was undertaken in an attempt to investigate the involvement of arginine metabolism on the IFN gamma induced anti-MHV3 effect in macrophages from resistant and susceptible mice.

NO, a product of arginine metabolism, is a highly reactive molecule produced from a guanidine nitrogen of arginine in a reaction catalyzed by NO synthase. It has been suggested that NO, which is synthesized in high levels by macrophages, plays a role in host defense. The NO is produced in response to stimulation by IFN gamma and may exert an antimicrobial activity against a variety of pathogens, providing a rapid and non-specific defense. [6, 10, 11, 13, 14, 18, 24, 33]. The arginase, an inducible enzyme in macrophages upon stimulation, has been implicated in virus inhibition and may potentially play an antimicrobial activity [4, 26, 31, 32, 39].

We show here that, in our culture conditions, both A/J and BALB/c mouse macrophages were not capable spontaneously to produce NO. Following stimulation with IFN gamma or IL4/IL10, the BALB/c mouse macrophages were always capable of synthesizing higher amounts of, respectively, NO or arginase, when compared to what we obtained with A/J mouse macrophages. As expected, when the cultures were performed in the presence of arginine free medium or NMA or both, a drastic decrease in the synthesis of NO was observed (Fig. 1). The MHV3 infection was capable to further enhance the NO release in both cell populations but did not influence the arginase expression, the lower levels observed in MHV3-infected BALB/c mouse macrophages being due to the cell destruction taking place during the virus infection (Fig. 1 and Fig. 2). Confirming previous reported data [26], the IFN gamma treatment had a small suppressive effect on the arginase expression in both macrophage populations. The partial inhibition of virus replication, which is induced by IFN gamma in A/J mouse macrophages, could not be reversed by macrophages treatment with NO or arginase inhibitors. Also, the MHV3 replication in IFN gamma activated or not activated BALB/c macrophages was not influenced by treatment with NO or arginase inhibitors.

Taken together the results show that both macrophage populations were capable of synthesizing NO and/or arginase but that the magnitude of MHV3 replication in these cells was not influenced by their induction or inhibition during the infection. In further support to this observation, we found that the level of MHV3 replication or inhibition was not influenced when we used macrophages from knockout mice for the iNOS gene, and as a consequence were unable of synthesizing NO (Table 1).

In conclusion, these data from the study of MHV3 replication and arginine metabolism modulated by cytokines like IFN gamma, IL4 and IL10 or inhibitors of the distinct pathways of such a metabolism, like NMA or OH-Arg, performed in macrophage cultures from MHV3-resistant and susceptible mice, led us to suggest that the antiviral state induced by IFN gamma occurring only in cells from resistant animals is not related to arginine metabolism. Such an evidence was reinforced by the results obtained with mice lacking inducible NO.

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