Studies on the Mechanism of the Interferon-mediated Antiviral State to Vesicular Stomatitis Virus in Resting Mouse Peritoneal Macrophages

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SUMMARY

We have analysed the expression of vesicular stomatitis virus (VSV) proteins in virus-infected freshly explanted mouse peritoneal macrophages (resistant to virus replication), macrophages aged in vitro (permissive for virus replication) and freshly explanted macrophages from mice treated with antibody to interferon (IFN) α/β (permissive for VSV replication). Our data showed that some VSV proteins (i.e. N/NS and G) were synthesized in virus-infected (1 p.f.u./cell) freshly harvested macrophages at early times after infection (3 to 6 h); the expression of such viral proteins was subsequently inhibited at 18 h post-infection. In contrast, a progressive increase in the expression of VSV proteins was observed in the macrophages aged in vitro and infected with VSV at 1 p.f.u./cell. Infection with a higher m.o.i. (16 p.f.u./cell) resulted in similar viral protein electrophoresis patterns for both aged macrophages and freshly explanted macrophages. Even at low m.o.i. a marked and progressive expression of all VSV proteins was observed in freshly harvested macrophages from mice treated with antibody to mouse IFN-α/β. Higher levels of oligo-2',5'-adenylate synthetase (2-5AS) were found in freshly harvested macrophages than in either aged macrophages or those from mice treated with antibody to IFN. No dsRNA-dependent 67K protein kinase was detected in freshly harvested macrophages or peritoneal cells from untreated mice or mice treated with poly(rI).poly(rC) or Newcastle disease virus. The following conclusions can be drawn from these results. Low levels of spontaneous IFN-α/β are responsible for the time-dependent inhibition of VSV protein synthesis in virus-infected freshly harvested macrophages; high levels of 2-5AS (in the absence of detectable levels of 67K protein kinase) appear to correlate with the progressive inhibition of VSV proteins; this natural antiviral state is highly effective only at low m.o.i.

Animal viruses do not generally multiply in mouse peritoneal macrophages when these cells have been freshly explanted, suggesting that this intrinsic antiviral activity may play an important role in the host defence against virus infections (Mogensen, 1979; Morahan et al., 1985). We had previously demonstrated that the resistance of resting mouse peritoneal macrophages to vesicular stomatitis virus (VSV) and encephalomyocarditis virus can be abolished by injection of mice with antibody to mouse interferon (IFN) α/β (Belardelli et al., 1984) or is spontaneously lost during cultivation in vitro of macrophages (Proietti et al., 1986; Gessani et al., 1987). Likewise, injection of mice with antibody to mouse IFN-α/β resulted in a marked decrease in the intracellular levels of oligo-2',5'-adenylate synthetase (2-5AS) activity in peritoneal macrophages (Gresser et al., 1985). These data suggested that low levels of IFN-α/β are spontaneously produced in normal mice, maintaining an antiviral state to VSV in peritoneal macrophages.

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IFNs can affect VSV replication in several ways. In fact, in different cell systems, VSV multiplication has been shown to be inhibited by IFN at the level of virus penetration (Whitaker-Dowling et al., 1983), mRNA cap methylation (De Ferra & Baglioni, 1981), mRNA transcription and translation (Belkowski & Sen, 1987), at the level of glycosylation of the envelope G glycoprotein (Olden et al., 1982) and intracellular transport of glycosylated (G) or membrane (M) proteins (Maheshwari & Friedman, 1985) or in various steps of final virus assembly (Jay et al., 1983; Maheshwari & Freidman, 1980).

We had previously demonstrated that binding and uptake of labelled VSV was similar in peritoneal macrophages from mice treated with antibody to IFN or from control mice, suggesting that virus entry was not significantly affected by the low levels of spontaneous IFN active on peritoneal macrophages (Belardelli et al., 1984). In the experiments reported here we have further investigated the possible intracellular mechanisms responsible for the IFN-mediated antiviral state to VSV in resting peritoneal macrophages and discuss the possible roles of the 2-5AS and 67K protein kinase in determining the intrinsic antiviral state to VSV of freshly harvested mouse macrophages.

Macrophages freshly harvested and aged in vitro were cultivated as described elsewhere (Belardelli et al., 1984) and infected with VSV at different m.o.i. At the indicated times, the cultures were labelled with [35S]methionine and, after a 1 h labelling period, the cells were processed to determine the amount of radioactive methionine incorporated in an acid-precipitable form and to assess viral protein production by SDS-PAGE (Laemmli, 1970). As shown in Fig. 1(a) some VSV proteins (i.e. N/NS and G) were synthesized in virus-infected (1 p.f.u./cell) freshly harvested macrophages at early times after infection (3 to 6 h); the expression of such viral proteins was subsequently inhibited at 18 h post-infection. Likewise, freshly explanted macrophages infected with VSV at the same m.o.i. did not exhibit any relevant inhibition in total cell protein synthesis (data not shown).

In contrast, a progressive increase in the expression of VSV proteins (Fig. 1a) and a concomitant decrease of host cellular protein synthesis (data not shown) were observed for aged macrophages infected at 1 p.f.u./cell. On the other hand, infection with VSV at high m.o.i. (16 p.f.u./cell) resulted in very similar electrophoresis patterns of virus proteins (Fig. 1b) and in a virtually identical inhibition of host protein synthesis (data not shown) for both freshly harvested and aged macrophages. Similar studies were performed on freshly harvested macrophages from mice treated with antibodies to mouse IFN-α/β. Seven-week-old male DBA/2 mice were treated intraperitoneally with 0.2 ml of either antibodies to IFN-α/β (1 × 10^5 neutralizing units per mouse) or normal sheep gamma globulin. Four days later, peritoneal cells from untreated or treated mice were harvested and infected with VSV (1 p.f.u./cell). The cultures were then labelled with [35S]methionine and the inhibition of total protein synthesis and VSV labelled proteins were analysed. In agreement with the results described above, the early expression of some VSV proteins was subsequently inhibited in freshly harvested macrophages from untreated mice (Fig. 2) or mice treated with normal sheep gamma globulin (data not shown). In contrast, a progressive increase in the expression of the five viral proteins was observed in VSV-infected macrophages from mice treated with antibody to IFN-α/β (Fig. 2). In addition, the host protein synthesis was strongly inhibited in VSV-infected macrophages from mice treated with antibody to IFN, compared to control macrophages (data not shown).

These results indicate that low levels of spontaneous IFN-α/β appear to be responsible for the time-dependent inhibition of VSV protein synthesis in virus-infected freshly explanted macrophages. In fact, a progressive increase of VSV protein expression occurred only in virus-infected macrophages from mice treated with antibody to IFN-α/β (Fig. 2), as well as in macrophage cultures permissive for virus replication (Fig. 1b). It is important to stress that the time-dependent inhibition of VSV proteins in virus-infected non-permissive macrophages was observed only at low m.o.i. (1 p.f.u./cell). In fact, infection of freshly harvested macrophages with VSV at higher m.o.i. (16 p.f.u./cell) resulted in a rapid shut-off of host protein synthesis and in the progressive expression of VSV proteins; this occurred similarly in virus-permissive aged macrophages. However, it is rather intriguing to observe that very low titres of VSV (10^3 to 10^4 p.f.u./ml) were recovered in the cell-free media of freshly harvested macrophages infected with a
Fig. 1. Gel autoradiographic patterns of uninfected and VSV-infected peritoneal macrophages. Peritoneal cells were harvested from 6-week-old male DBA/2 mice and peritoneal macrophages were cultivated as previously described (Belardelli et al., 1984). Freshly harvested macrophages (3 h after seeding) and aged mouse macrophages (4 days of culture in vitro) (left- and right-hand parts respectively) were infected with VSV at 1 p.f.u./cell (a) and 16 p.f.u./cell (b) and labelled for 1 h with [35S]methionine (885 Ci/mmol; final concentration of 15 to 20 µCi/well). Equal amounts of radioactive proteins (10⁶ c.p.m. of acid-precipitable material) of each sample were analysed at the different times indicated (h) after virus infection, on 8.5% polyacrylamide gels according to Laemmli (1970). 0, uninfected cells. L, G, N, NS and M are the viral polypeptides. Numbers at the right of the gels indicate the $M_r$ values of radiolabelled standard proteins (Amersham).
Fig. 2. Viral protein production in VSV-infected peritoneal macrophages from untreated mice or mice injected with antibodies to IFN-α/β. Seven-week-old male DBA/2 mice were injected intraperitoneally with 0.2 ml of antibody to IFN-α/β. Four days later, the peritoneal macrophages were seeded in 24-well plastic plates and infected with VSV (1 p.f.u./cell). The peritoneal macrophages from untreated mice (a) or anti-IFN-treated mice (b) were labelled with [35S]methionine and protein patterns were analysed as described in Fig. 1.

high m.o.i. of VSV, in spite of the marked expression of virus proteins (Fig. 1b). In this regard it is possible to speculate that resting macrophages exhibit additional antiviral mechanisms that can also affect late steps of the VSV replication cycle, including maturation and release of infectious particles.

The phenomenon of early expression of some VSV proteins (followed by the subsequent inhibition of protein synthesis) suggested that the antiviral state to VSV in freshly harvested macrophages was not likely to be mediated by alterations in the very early steps of the virus–cell cycle interaction, such as virus uptake and early expression of VSV RNA. Degradation of virus RNA and/or inhibition of VSV RNA translation could be considered as possible mechanisms for the subsequent inhibition of virus protein synthesis occurring in virus-infected resting macrophages. In order to clarify the possible role(s) of the two major IFN-induced enzymes (i.e. 2–5AS and 67K protein kinase) on the IFN-induced inhibition of VSV proteins in resting macrophages, we have analysed their levels in virus-non-permissive and virus-permissive macrophage cultures. In agreement with previous observations (Proietti et al., 1986), cell extracts from macrophages aged in vitro from DBA/2 mice exhibited five- to eightfold less 2–5AS activity with respect to freshly harvested macrophages (data not shown). Likewise, cell extracts from mice treated with antibody to IFN-α/β exhibited much lower amounts (five- to sixfold less) of 2–5A oligomers than did extracts of macrophages from control mice (data not shown).

To evaluate the levels of dsRNA-dependent 67K protein kinase activity in macrophage extracts, this enzyme was measured in solution, as previously described (Romeo et al., 1985). No characteristic dsRNA-dependent 67K phosphorylated band was observed in freshly harvested
Fig. 3. Autoradiography of SDS-polyacrylamide gel of phosphorylation reaction products (from kinase assay in solution) from S-10 cell-free extracts of peritoneal macrophages. Peritoneal macrophages were harvested and seeded in plastic dishes as described (Belardelli et al., 1984). L929 cells were seeded at 3.5 x 10^4 cells/cm^2 1 day before the addition of mouse IFN-α/β (200 units/ml) for 20 h. S-10 cell extracts, assay of protein kinase activity (in solution) and SDS-PAGE were performed as previously described elsewhere (Romeo et al., 1985). The patterns of phosphorylation are shown in the absence (even-numbered lanes) or presence (odd-numbered lanes) of dsRNA poly(rI), poly(rC). The position of the 67K polypeptide is indicated by the arrowheads and M_r markers [bovine serum albumin (68K) and chymotrypsinogen (25K)] are also indicated. Assay mixtures applied to each lane were as follows. (a) Lane 1, L929 + IFN-α/β; lanes 2 and 3, freshly harvested peritoneal macrophages (3 h after cell seeding). (b) Lane 1, L929 + IFN-α/β; lanes 2 and 3, freshly explanted total peritoneal cells from control mice; lanes 4 and 5, total peritoneal cells from mice treated with poly(rI), poly(rC) (100 μg per mouse intravenously, 24 h before harvesting of peritoneal cells); lanes 6 and 7, total peritoneal cells from mice treated with NDV (160 haemagglutination units per mouse intravenously, 24 h before harvesting of the peritoneal cells).

macrophages (Fig. 3a); such a band was clearly visualized in cell extracts from L929 cells treated with IFN-α/β. The absence of detectable enzyme activity in macrophage extracts was also confirmed when the kinase activity was measured after immobilization on poly(rI), poly(rC)-agarose (data not shown). Fig. 3(b) shows the analysis performed on extracts of total peritoneal cells from control mice (lanes 2 and 3) or from mice treated with IFN inducers, such as poly(rI), poly(rC) (lanes 4 and 5) or Newcastle disease virus (NDV) (lanes 6 and 7). In no instance was expression of the typical 67K phosphorylated band observed in the cell extracts from macrophages.

These results indicate that high levels of 2-5AS (in the absence of any detectable dsRNA-dependent 67K protein kinase) appear to correlate with the progressive inhibition of virus protein synthesis and the general antiviral state to VSV in freshly harvested macrophages. This finding does not necessarily imply that 2-5AS plays a crucial role in the natural antiviral state to VSV of peritoneal macrophages. In fact, there is some controversy in the literature concerning the role of the two major IFN-induced cellular enzymes in the inhibition of VSV replication. Based on data indicating that a drastic inhibition of VSV protein synthesis occurs in IFN-treated cells that contain significant amounts of VSV mRNA, some authors have concluded that the 2–5A-mediated degradation of VSV mRNAs is not responsible for the inhibition of viral protein synthesis in human cells; they suggested an involvement of the 67K protein kinase in the IFN-induced inhibition of VSV replication (Masters & Samuel, 1983). In contrast to these findings, other investigators showed that several human and murine cell lines can develop an
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IFN-mediated antiviral state to VSV with a strong induction of 2–5AS, in the absence of any protein kinase activity (Sen & Herz, 1983; Holmes & Gupta, 1982; Banerjee et al., 1983). In addition Lewis (1988) has recently shown that the induction of an antiviral state to VSV by IFN can be established in the absence of both 2–5AS and 67K protein kinase.

At the present time we speculate that, in addition to the 2–5AS pathway, other intracellular mechanisms (different from the 67K protein kinase) may be responsible for the inhibition of virus protein synthesis in virus-non-permissive macrophages infected with VSV at low m.o.i.; additional unknown antiviral mechanisms (acting at late stages of the virus cycle) could restrict viral production, even when macrophages are challenged with a high m.o.i. and virus protein synthesis is fully expressed in these cells.

Lastly, we emphasize that freshly explanted resting macrophages can represent a useful cell model to study the mechanisms of their intrinsic antiviral activity and the role(s) of extrinsic factors such as endogenous IFNs, constitutively expressed under physiological conditions.

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