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Inhibitory effects of EICAR on infectious pancreatic necrosis virus replication

M. Jashés [a], G. Mlynarz [a], E. De Clercq [b], A.M. Sandino [a,*]

[a] *Laboratorio de Virología, Departamento de Ciencias Biológicas, Facultad de Química y Biología, Universidad de Santiago de Chile, PO Box 40, Alameda 3363, Santiago 33, Chile*

[b] *Rega Institute for Medical Research, Katholicke Universiteit Leuven, B-3000 Leuven, Belgium*

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Abstract

Recently, the antiviral 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide (EICAR) was shown to inhibit the replication of the infectious pancreatic necrosis virus (IPNV). In order to obtain more information about the mechanism of the antiviral action of EICAR we studied its effect on viral macromolecules synthesis. EICAR was found to inhibit IPNV messenger and genomic RNA synthesis. To inhibit viral RNA synthesis, EICAR must be added at least 3 h before the start of RNA synthesis. This suggests that EICAR does not directly affect the viral RNA polymerization process. Moreover, the antiviral action of EICAR was reversed by the exogenous addition of guanosine (5–50 μg/ml), but not adenosine or cytidine (10–100 μg/ml). Our findings suggest that the antiviral action of EICAR is mediated by a reduction of the intracellular guanosine 5′-triphosphate (GTP) pool level, as has been observed with ribavirin and EICAR in other biological systems.

Keywords: EICAR; IPNV; GTP pool level

1. Introduction

In the last years, various antiviral derivatives of ribavirin have been synthesized. One of the most potent compound was 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide (EICAR; Fig. 1). EICAR inhibits the "in vitro" replication of a diversity of viruses such as pox-, toga-, arena-, reo-, orthomyxo- and paramyxoviruses. Its antiviral potency is approximately ten to 100 times greater than that of ribavirin (De Clercq et al., 1991; Shigeta et al., 1992).

The action mechanism of EICAR is unknown, but it seems to be similar to that of ribavirin (De Clercq et al., 1991; Balzarini et al., 1993). Ribavirin has been identified as an inhibitor of inosine 5′-monophosphate dehydrogenase (IMP dehydrogenase), the enzyme that converts inosine 5′-monophosphate (IMP) to xanthosine 5′-monophosphate (XMP). Consequently, the intracellular pools of guanosine 5′-monophosphate (GMP), guanosine 5′-diphosphate (GDP) and guanosine 5′-triphosphate (GTP) are reduced and viral RNA synthesis is suppressed (Streeter et al.,

* Corresponding author. Fax: +56-2-6812108.

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1973). The fact that the antiviral effect of ribavirin is readily reversed, following the exogenous addition of guanosine (Scholtissek, 1976), indicates that it may indeed act via depletion of the GTP pool levels. Similarly, it has been demonstrated by a plaque inhibition assay that the inhibitory effect of EICAR on several viruses (including respiratory syncytial virus, parainfluenza, reovirus, vesicular stomatitis virus and sindbis virus) is reversed following an exogenous addition of guanosine (De Clercq et al., 1991). EICAR also inhibits the proliferation of murine leukemia L1210 cells and human lymphocyte CEM cells (Balzarini et al., 1993). This antitumoral effect is caused by rapid and marked inhibition of IMP dehydrogenase activity, in both cell-free systems and intact cells. Also, the 5′monophosphate of EICAR was found to inhibit purified L1210 IMP dehydrogenase.

Recently, we demonstrated that EICAR inhibits the replication of infectious pancreatic necrosis virus (IPNV), which is one of the most important salmon pathogens. It causes a serious disease in trout and salmon farms around the world. The EICAR concentration required to inhibit IPNV plaque formation by 50% (EC_{50}) is 0.01 μg/ml, which means that IPNV is 20–400 times more susceptible to this antiviral compound than the other viruses tested (Jashés et al., 1996). This makes it a good candidate to be used in an anti-IPNV therapy.

IPNV has a bisegmented double-stranded RNA (A and B) as a genome, and it belongs to the *Birnaviridae* family (Dobos et al., 1979; Dobos and Roberts, 1982). The RNA A segment encodes a polyprotein of 106 kDa, which is co-translationally processed, leading to the polypeptides VP2, VP3 and VP4. The RNA B segment encodes VP1, the highest molecular weight peptide, which can be free in the virion (called VP1) probably as the viral polymerase. It also is covalently bound to the 5′ end of both RNA segments as a VPg. In respect to the viral macromolecular synthesis, it has been determined that the IPNV RNA messenger synthesis begins approximately at 3 h post-infection. The viral polypeptides are synthesized in a syncronic manner starting at 6 h post infection and the genomic RNA is observed at 8–10 h post-infection (Dobos, 1977; Somogyi and Dobos, 1980).

In order to get a broader insight into the action mechanism of EICAR against IPNV replication, we examined whether the inhibitory effect of EICAR on IPNV is caused by the inhibition of viral RNA synthesis and if this inhibitory effect is related to a depletion of the intracellular GTP pool. For this purpose, we studied the effect of EICAR on IPNV macromolecules synthesis during IPNV infection of CHSE-214 cells.

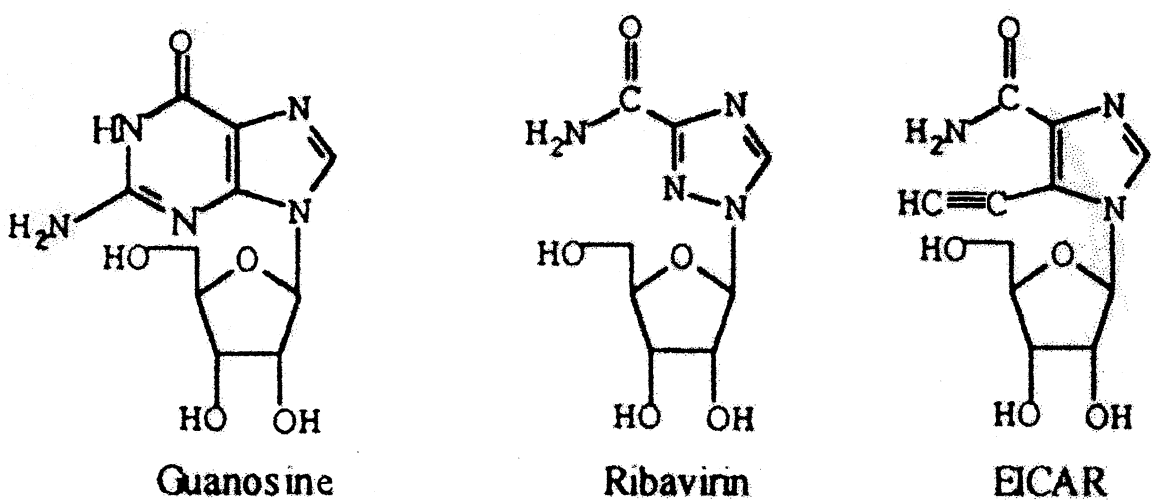

Fig. 1. Structure of the antiviral compounds: guanosine analogs.

2. Materials and methods

2.1. Viruses and cells

Infectious pancreatic necrosis virus, IPNV (VR-299 strain), was grown in monolayers of the Chinook salmon embryo cell line (CHSE-214). The cells were infected with 0.1 plaque forming units (pfu) per cell. They were grown in minimal Eagle's medium (MEM) supplemented with 2% fetal calf serum, 100 UI/ml penicillin, 100 UI/ml streptomycin, 25 UI/ml nystatin and 10 mM HEPES pH 7.3. After viral adsorption at 15°C for 1 h, the cells were incubated until a cytopathic effect greater than 90% was observed (approximately 3 days). The viral inoculum was stored in aliquots at −20°C.

2.2. EICAR's effect on IPNV RNA synthesis

Since IPNV genome RNA can be detected from about 8 to 10 h post-infection (h pi), the effect of EICAR on viral genomic RNA synthesis was evaluated at 10 h pi (Somogyi and Dobos, 1980; Jashés, 1996). CHSE-214 cells (6×10^5) were infected at a m.o.i. of 50. After viral adsorption several concentrations of EICAR plus 50 μCi [^{32}P]orthophosphoric acid were added, and then incubated until 10 h pi. All the nucleic acid was extracted from the whole monolayer cell. Then it was precipitated, analyzed by 7% polyacrylamide gel electrophoresis (PAGE) and autoradiography. EICAR was added when viral adsorption was finished, corresponding to the 0-h pi. Variations with respect to both drug concentration and the time at which it was added are specified in the results.

2.3. EICAR's effect on viral transcription: monitoring of viral polypeptide expression

The effect of EICAR on IPNV viral transcription was determined by monitoring viral polypeptide expression according to the above protocol with the following modifications. The polypeptides were intracellularly labeled by a pulse of 50 μCi/ml [^{35}S]methionine during 2 h from 6 to 8 h pi, when viral polypeptide synthesis begins. It was analyzed by resuspending the whole cell monolayer in a disruption protein buffer. The total preparation was loaded and samples were analyzed by 12% SDS-PAGE and autoradiography.

2.4. The effect of nucleoside addition on EICAR's inhibition of IPNV

The effect of exogenous nucleoside addition on the inhibition by EICAR of either viral genomic RNA synthesis or viral polypeptide expression was analyzed as described. In these experiments 0.5 μg/ml of EICAR plus guanosine at 5, 10, 50 μg/ml, or either adenosine or cytidine at 10, 50, 100 μg/ml were added. At the same time 50 μCi [^{32}P]orthophosphoric acid or [^{35}S]methionine was added to study the synthesis of genomic RNA or viral polypeptides, respectively. Viral macromolecules synthesis was analyzed by electrophoresis and autoradiography.

The experiments from Section 2.2 to Section 2.4 were performed at least three times. The results shown in the following figures are representative of the results of these experiments.

3. Results

3.1. The effect of EICAR on IPNV RNA replication and viral polypeptide expression during viral infection

Our previous results showed that the concentration required for EICAR to inhibit IPNV plaque formation by 50% (EC_{50}) is 0.01 μg/ml. Decrease of cellular DNA synthesis by 50% (IC_{50}) and reduction of the cellular protein synthesis by 10% can be observed by the application of 1 and 5 μg/ml EICAR, respectively (Jashés et al., 1996). Therefore, in this study EICAR was tested within the concentration range of 0.1–1 μg/ml. Fig. 2 shows that EICAR inhibits genomic RNA synthesis even at a concentration of 0.1 μg/ml (lane 3). At higher concentrations the inhibitory effect becomes more pronounced (lane 5), and at 1 μg/ml viral genomic RNA synthesis could no longer be detected (lane 7). Similar effects were observed when RNA synthesis was analyzed at 26.5 h pi

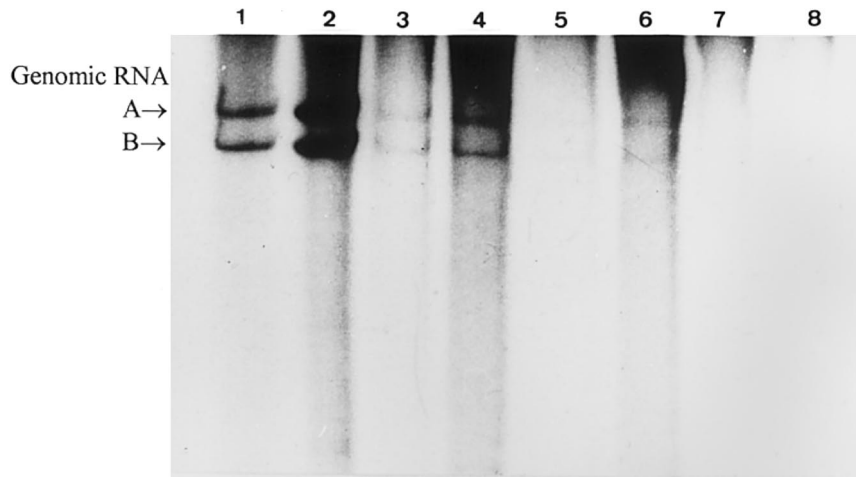

Fig. 2. The effect of EICAR on IPNV genomic RNA synthesis. Monolayers of CHSE-214 cells were infected with IPNV either in the absence (lanes 1 and 2) or presence of EICAR at 0.1, 0.5 and 1.0 μg/ml (lanes 3 and 4; 5 and 6; 7 and 8, respectively). EICAR and 50 μCi/ml of [^{32}P]orthophosphoric acid were added simultaneously at 0 h pi and the cells were incubated at 15°C. At 12 h pi (lanes 1, 3, 5 and 7) or at 26.5 h pi (lanes 2, 4, 6 and 8) the cells were harvested, the RNA extracted and analyzed by 7% PAGE and autoradiography. The migration of the A and B segments of the IPNV genomic RNA is indicated by arrows.

(lanes 4, 6 and 8). The identification of genomic RNA segments was performed using extracted RNA from purified virus stained with silver nitrate as an electrophoretic migration control.

In order to evaluate the EICAR effect on the viral messenger RNA, an indirect analysis was carried out. The EICAR effect on the viral transcription process was made monitoring the viral polypeptide expression (Fig. 3). This analysis was possible because the concentration of EICAR used was much lower than those required to affect cellular protein synthesis (De Clercq et al., 1991; Jashés, 1996). In addition, IPNV polypeptide detection was easier than viral messenger RNA detection. IPNV messenger RNA could only be detected using a highly radioactive probe and a lot of infected cell numbers.

Since the viral polypeptides VP2, VP3 and VP4 are synthesized from a polyprotein, the detection of any of them can be used to follow the appearance of all of them (Duncan and Dobos, 1986). This is very useful because VP2 comigrates with cellular polypeptides which makes difficult its observation (Dobos, 1977) and VP4 decreases during the infection due to the generation of truncated VP4 forms (Maygar and Dobos, 1994). Instead the observation of VP3 accumulation is easy, which allows for the analysis of the effect of EICAR on the viral messenger synthesis. Lane 2 (Fig. 3) shows the expression of the viral polypeptide VP2, VP3 and VP4 in the absence of EICAR. To identify the electrophoretic migration of the viral polypeptides, purified viruses labelled with ^{35}S methionine were used as a migration standard. When EICAR was added, polypeptides synthesis decreased even at an EICAR concentration of 0.1 μg/ml (lane 3). At higher antiviral concentrations the inhibition became more pronounced (lanes 4 and 5). These results suggest that the viral messenger RNA synthesis was inhibited by EICAR.

3.2. The effect of EICAR on genomic RNA synthesis and viral polypeptide expression when it was added at different times after the viral infection

EICAR was added at different times after infection taking into account that the IPNV messenger RNA synthesis starts at 3–4 h pi, the viral polypeptide synthesis at 6–8 h pi and the genomic synthesis at 8–10 h pi (Dobos, 1977; Somogyi and Dobos, 1980; Jashés, 1996). Assuming that 1 μg/ml of EICAR affects cellular DNA synthesis in these studies 0.5 μg/ml of this compound was

added at: (i) 3 h before IPNV infection (− 3 h pi); (ii) immediately after virus adsorption (0 h pi); (iii) at the start of viral transcription and approximately 4 h before viral RNA replication has started (4 h pi); and (iv) nearly at the start of viral RNA replication (7 h pi). Fig. 4 shows the effect of EICAR on genomic RNA synthesized until 10 h pi. Inhibition of viral RNA replication by EICAR was seen only when it was added previously to infection (lane 1), immediately after viral adsorption (lane 2), and at 4 h pi (lane 3). No effect was observed when it was added close to the start of RNA replication at 7 h pi (lane 4).

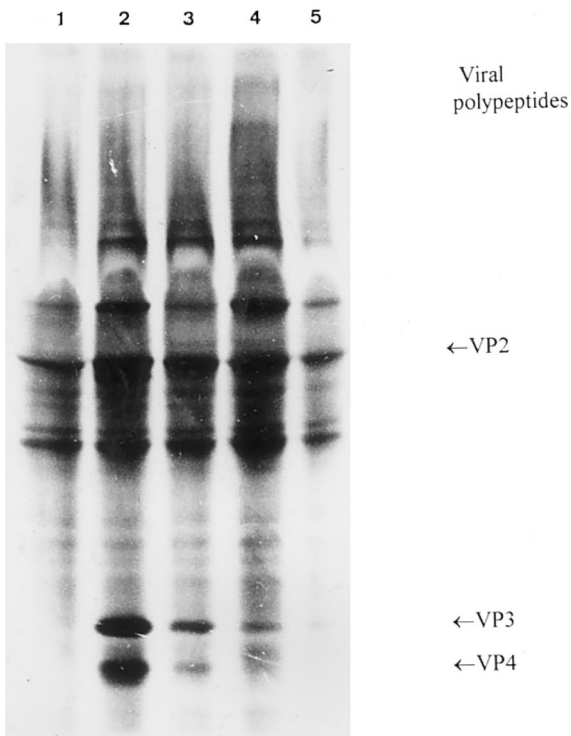

Fig. 3. The effect of EICAR on IPNV polypeptide expression. Monolayers of CHSE-214 cells were infected with IPNV either in the absence (lane 2) or the presence of EICAR at 0.1, 0.5 and 1.0 μg/ml (lanes 3, 4 and 5 respectively). The antiviral compound was added at 0 h pi, the cells were incubated at 15°C, and at 6 h pi a radioactive pulse of 50 μCi/ml of [^{35}S]methionine was applied. At 8 h pi 100 μl of a protein disruption solution was added on the monolayer. The polypeptides were analyzed by 12% SDS-PAGE and autoradiography. The control of non-infected cells was also included in lane 1. The migration of IPNV polypeptides is indicated.

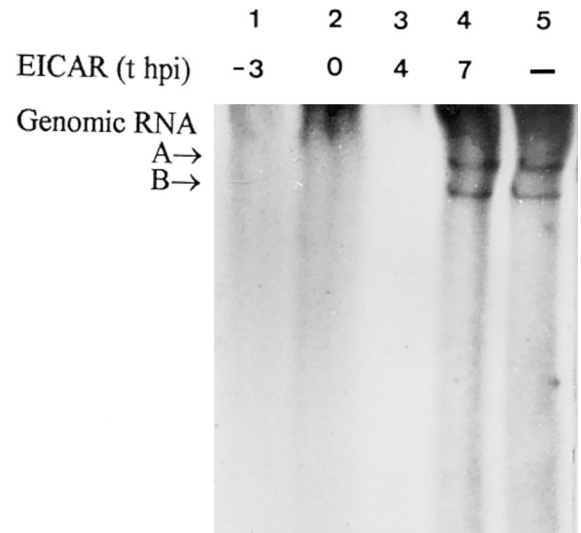

Fig. 4. EICAR addition at different times during the IPNV infective cycle. The effect on viral genomic RNA synthesis. Monolayers of CHSE-214 cells were infected with IPNV either in the absence (lane 5) or in the presence of EICAR (0.5 μg/ml) which was added at different times after infection (*t* h pi): − 3 h pi (that is 3 h prior to infection), 0, 4 and 7 h pi. In all cases 50 μCi/ml of [^{32}P]orthophosphoric acid were added at 0 h pi and the cells were incubated at 15°C. At 10 h pi the cells were harvested and the RNA was extracted and analyzed by 7% PAGE and autoradiography. The migration of the A and B segments of the IPNV genomic RNA is indicated by arrows.

The effect of EICAR on viral polypeptide synthesis from 6 to 8 h pi is shown in Fig. 5. The greatest inhibition was observed when EICAR was added at 0 h pi (lane 6), or even previously to infection (lane 4). EICAR could not prevent the synthesis of viral polypeptides, particularly those that are easily detected (VP3 and VP4) when it was added at 4 h pi (lane 8). In similar experiments we observed that viral polypeptide synthesis occur even when EICAR was added at 2 h pi (data not shown). Thus, viral messenger RNA synthesis is only affected when EICAR is added a few hours before viral transcription starts. Furthermore, genomic RNA synthesis is inhibited by adding EICAR at 4 h pi (Fig. 4, lane 3) while viral transcription is not affected (viral polypeptide synthesis; Fig. 5, lane 8). These results indicate that the inhibition of genomic RNA replication does not occur as a consequence of viral transcription inhibition.

3.3. The effect of exogenous nucleoside application on IPNV inhibition by EICAR

In order to study the influence of guanosine on the inhibitory effect of EICAR on both genomic RNA synthesis and viral polypeptide expression, 0.5 μg/ml of EICAR was added to infected cells at 0 h pi, either in the absence or the presence of 5, 10 or 50 μg/ml of guanosine. As in the other assays, the genomic RNA synthesis was analyzed until 10 h pi, and viral polypeptide expression from 6 to 8 h pi. The inhibition caused by EICAR on IPNV genomic RNA synthesis was reversed by guanosine (Fig. 6, lanes 3–5). In addition, 10 or 50 μg/ml of guanosine (Fig. 7, lanes 2 and 3) re-establish viral polypeptide synthesis as compared to the control (lane 1). Similar experiments were done using either adenosine or cytidine to evaluate the specificity of the guanosine effect. As shown in Fig. 8, an addition of adenosine or cytidine at 10, 50, 100 μg/ml did not affect viral genomic RNA synthesis (lanes 3–5 and 6–8, respectively). Moreover, in infected monolayers the cytopathic effect of IPNV was also observed when the infection was carried out in the presence of both EICAR and guanosine. No cytopathic effect was observed when EICAR alone or EICAR with either adenosine or cytidine were added. Similar

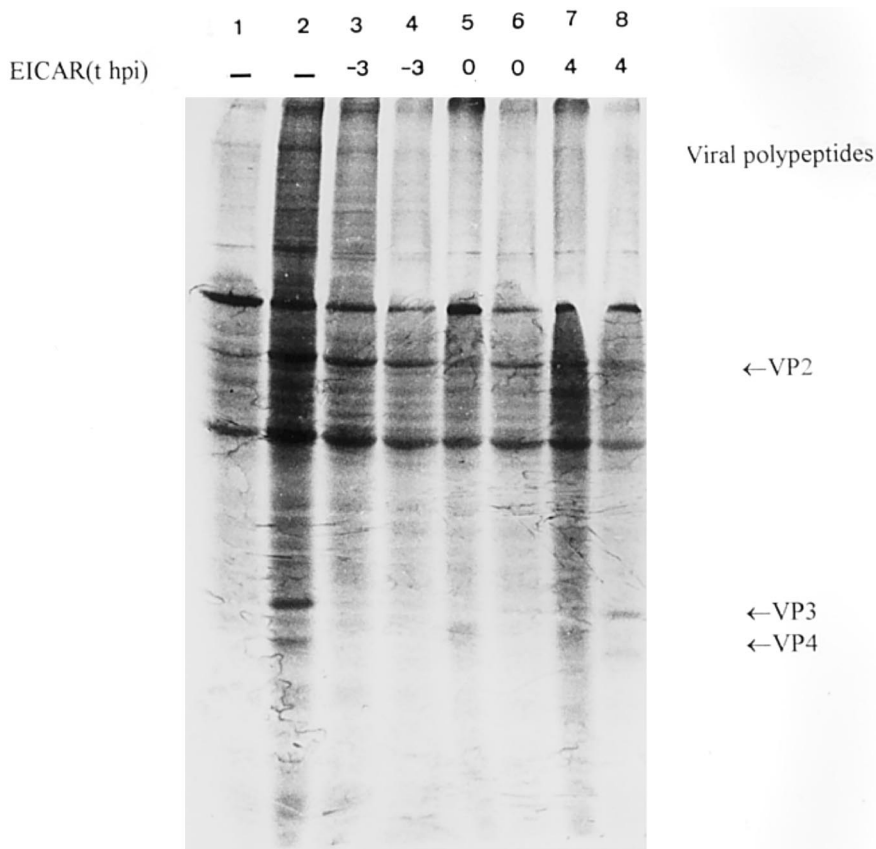

Fig. 5. EICAR addition at different times during the IPNV infective cycle. The effect on viral polypeptide expression. IPNV-infected (lanes 2, 4, 6 and 8) or uninfected (lanes 1, 3, 5 and 7) CHSE-214 cells were exposed to EICAR (0.5 μg/ml) which was added at several times after infection: − 3 h pi (that is 3 h prior to infection), 0 and 4 h pi. The antiviral compound was not added to samples of lanes 1 and 2. In all cases they were incubated at 15°C and a pulse of 50 μCi/ml of [^{35}S]methionine was applied at 6 h pi. Finally the medium was removed at 8 h pi and 100 μl of a protein disruption solution was added on the monolayer. The polypeptides were analyzed by 12% SDS-PAGE and autoradiography. The migration of IPNV polypeptides is indicated.

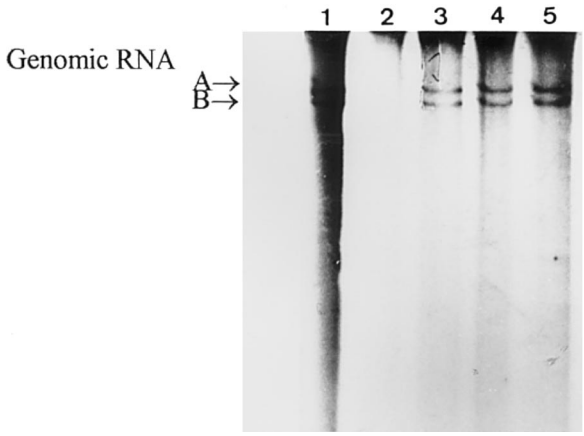

Fig. 6. The effect of guanosine addition on the inhibition of IPNV RNA replication by EICAR. Monolayers of CHSE-214 cells were infected and at 0 h pi 0.5 μg/ml of EICAR plus 50 μCi/ml of [^{32}P]orthophosphoric acid were added either in the absence (lane 2) or the presence of guanosine at 5 μg/ml (lane 3), 10 μg/ml (lane 4), or 50 μg/ml (lane 5). Infected cells without EICAR and guanosine are shown in lane 1. All the samples were incubated at 15°C. At 10 h pi the cells were harvested and the RNA was extracted and analyzed by 7% PAGE and autoradiography. The migration of the A and B segments of the IPNV genomic RNA is indicated by arrows.

results were obtained when viral polypeptide synthesis was analyzed (data not shown).

4. Discussion

Our results clearly indicate that EICAR inhibits IPNV genomic and messenger RNA synthesis. This inhibition is both concentration- and time-dependent. EICAR is more effective before RNA synthesis starts, that is either immediately after the viral adsorption or before the infection. On the other hand, an addition of EICAR at 7 h pi has no effect on genomic RNA synthesis (which starts after 8 h pi). These results suggest that EICAR has no direct effect on the viral RNA polymerase. However we cannot exclude that in the triphosphate form, EICAR also might affect the viral RNA polymerase, as described for ribavirin triphosphate, in the case of the influenza virus (Wray et al., 1985; Gilbert and Knight, 1986). It would be interesting to evaluate the effect of EICAR on IPNV in an "in vitro" transcription assay to verify this hypothesis, but such experiments would require an available triphosphorylated form of EICAR (Zimmerman and Deeprose, 1978; Stridh, 1983; Balzarini et al., 1993).

It has been previously demonstrated that ribavirin's antiviral activity is due to a depletion of the intracellular GTP levels required for viral RNA transcription and replication. Therefore, the antiviral effects of ribavirin can be reversed by the addition of either guanosine or xanthosine (which

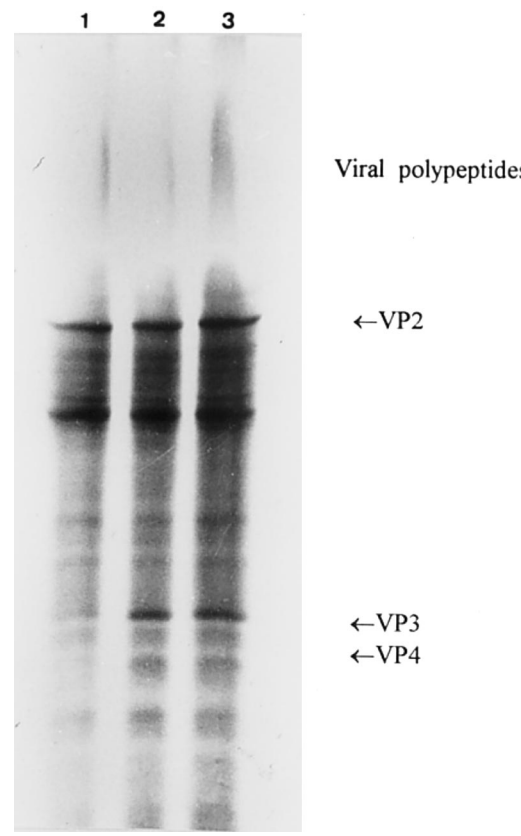

Fig. 7. The effect of guanosine addition on the inhibition of IPNV polypeptide expression by EICAR. Monolayers of CHSE-214 cells were infected and EICAR (0.5 μg/ml) was added at 0 h pi either in the absence (lane 1) or the presence of guanosine at 10 or 50 μg/ml (lanes 2 and 3, respectively). Then they were incubated at 15°C, and at 6 h pi 50 μCi/ml of [^{35}S]methionine was added. At 8 h pi the culture medium was removed and 100 μl of a protein disruption solution was added. Samples were analyzed by 12% SDS-PAGE and autoradiography. The migration of IPNV polypeptides is indicated.

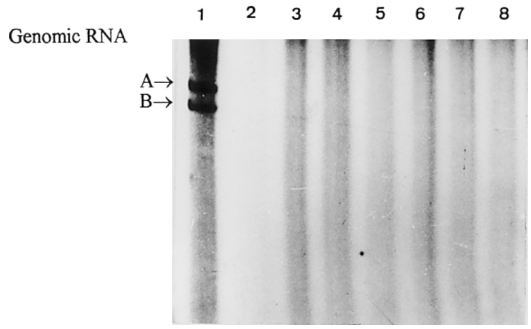

Fig. 8. The effect of adenosine or cytidine addition on the inhibition of IPNV RNA replication by EICAR. CHSE-214 cells monolayers were infected, at 0 h pi 50 μCi/ml of [^{32}P]orthophosphoric acid were added in the presence of: EICAR (0.5 μg/ml; lane 2); EICAR plus adenosine at 10, 50, 100 μg/ml (lanes 3, 4, 5, respectively); or EICAR plus cytidine at 10, 50, 100 μg/ml (lanes 6, 7, 8, respectively). Lane 1 corresponds to the infected monolayer cells without EICAR. Then the cells were incubated at 15°C and at 10 h pi the cells were harvested, the RNA was extracted and analyzed by 7% PAGE and autoradiography. The migration of the A and B segments of the IPNV genomic RNA is indicated by arrows.

are used for guanosine nucleotides synthesis). Other nucleosides such as adenosine or cytidine, which cannot be used for guanosine nucleotide synthesis, do not reverse this inhibitory effect. This is due to the fact that ribavirin blocks the enzymatic reaction catalyzed by IMP dehydrogenase, which converts inosine monophosphate to xanthosine monophosphate (Scholtissek, 1976; De Clercq et al., 1991; Balzarini et al., 1993). As described for other viruses, we also found that the EICAR inhibitory activity was reversed by an addition of guanosine (De Clercq et al., 1991). We also found that the addition of guanosine reverts the inhibitory effect of EICAR on viral RNA synthesis. Nevertheless, the inhibitory effect of EICAR on viral RNA synthesis was not affected by adding adenosine or cytidine. This indicates that the mechanism by which EICAR causes IPNV inhibition is associated with the guanine nucleotide biosynthetic pathway. If we take into account the EICAR results obtained by Balzarini et al. (1993) the results observed in our work are probably due to a depletion in the intracellular GTP pool level, which occurs as a consequence of IMP dehydrogenase inhibition by EICAR. Moreover, the guanine nucleotide synthesis is restarted when guanosine is added, which probably occurs through the rescue or the alternative pathway. To further corroborate this mechanism it is necessary to determine the intracellular levels of the guanine nucleotides and IMP in IPNV-infected CHSE-214 cells.

The high susceptibility of IPNV to EICAR may reflect the important role of GTP in the beginning of IPNV RNA synthesis. It has been described that during RNA synthesis the IPNV VP1 protein may function as a primer. It adds guanosine monophosphates sequentially in a template dependent reaction to form VP1pGpG complexes (Dobos, 1993, 1995). Thus, the intracellular availability of GTP may be crucial for the start of IPNV RNA synthesis, and this would explain the significant impact of EICAR on IPNV replication.

Acknowledgements

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