INHIBITION OF RNA-DEPENDENT DNA POLYMERASE OF ONCORNA VIRUSES BY CARBOPOL 934

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1. Introduction

De Clercq and Claes [1] reported the stimulation of RNA-dependent DNA polymerase (reverse transcriptase) of the Moloney strain of Murine Leukemia Virus M-MuLV) by Carbopol 934 (according to the suppliers a cross-linked polyacrylic acid with a mol. wt of approx. 3×10^6). The reported stimulation is 8-fold at a Carbopol concentration of 160 μg/ml. These authors [1] recommend Carbopol as 'a valuable tool for increasing the sensitivity of detection of reverse transcriptase activity in oncogenic RNA viruses and malignant tissues'. However, it appears from the data presented in this paper, that stimulation of enzymatic activity by Carbopol is not a property shared by all reverse transcriptases, on the contrary, we usually observed inhibition by Carbopol of reverse transcriptase of Rauscher Murine Leukemia Virus R-MuLV) and Avian Myoblastosis Virus (AMV).

This result is in agreement with the inhibition of various reverse transcriptases and other nucleic acid polymerases by pyran copolymers carrying maleic anhydride groups which, in the presence of H_2O, become hydrolyzed to the corresponding carboxylic acids [2]. As discussed by Papas et al. [2] pyran copolymers possess immunostimulating [3], interferon inducing [4], and antiviral and antitumor activities [5,6]. Likewise, Carbopol inhibits the induction of leukemia by R-MuLV in Balb/c mice (to be published elsewhere), although it is not yet clear that the latter effect is indeed mediated by inhibition of reverse transcriptase.

2. Materials and methods

2.1. *Materials*

Avian Myoblastosis Virus (AMV) was a gift from Dr W. J. Beard, Life Sciences Building, St. Petersburg, Florida, USA. Reverse transcriptase from AMV was prepared by L. H. Cohen of our laboratory as described by Verma and Baltimore [7].

Reverse transcriptase activity of Rauscher Murine Leukaemia Virus (R-MuLV) was tested in freshly prepared virus pellets. The JLS-V9 cell line, derived from bone marrow cells of Balb/c mice infected with and producing R-MuLV [8], was grown in monolayers in Eagle's minimal essential medium completed with 10% calf serum.

24 hr old culture fluids were clarified by centrifugation during 10 min at 20 000 g at 4° C and subsequently layered over 2 ml 50 mM Tris–HCl pH 7.8, 100 mM KCl in 20% glycerol and centrifuged in a Spinco 50-Ti rotor during 30 min at 50 000 rev/min at 4° C. The virus pellets were resuspended in 200 μl of a buffer containing 50 mM Tris–HCl pH 7.8, 100 mM NaCl, 1 mM dithiothreitol and 0.1% Triton X-100 and either used immediately or stored at −20° C until use. Carbopol 934 was a gift from B. F. Goodrich, Chemical Cy., Cleveland, Ohio, USA. It was dissolved at a concentration of 4 mg/ml in saline and neutralized as described by De Clerq and Claes [1]. [^{3}H]dGTP and [^{3}H] dTTP were obtained from the Radiochemical Centre, Amersham, England. Poly-A and poly-C were purchased from Sigma Chemical Cy., St. Louis, Missouri, USA. Oligo-dG 12–18 and oligo-dT 12–18

were the products of Collaborative Research, Waltham, Massachusetts, USA.

2.2. *Assay of reverse transcriptase activities*

Unless specified otherwise, enzyme activities were assayed as follows:

a) Endogenous activity: 20 μl of freshly R-MuLV suspension were incubated at 37°C in a final vol of 50 μl in the presence of the following components (final concentrations): Tris–HCl pH 7.8 (40 mM), NaCl (90 mM), $MnCl_2$ (500 μM), dithiothreitol (4 mM), Triton X-100 (0.015%), dATP, dCTP and dTTP (130 μM each), [^{3}H]-dGTP (13 μM and 550 Ci/mol) and Carbopol as indicated. At appropriate times, 50 μl of carrier yeast RNA (0.5 mg/ml) was added and the samples were transferred to 5% trichloroacetic acid in 10 mM sodium pyrophosphate. Acid precipitable radioactivity was collected on Millipore filters and counted in a Packard Tri-carb. Liquid Scintillation Spectrometer. The data were corrected by substraction of a blank (assay without enzyme preparation) which varied between 50 and 100 cpm.

b) Poly-A or poly-C dependent activities: 10 μl of enzyme preparations, either derived from 7 ml of culture fluids containing R-MuLV or from AMV, were incubated at 37° C in a final volume of 60 μl in the presence of the following components (final concentrations): Tris–HCl pH 7.8 (60 mM), NaCl (45 mM), KCl (60 mM), $MnCl_2$ (500 μM), dithiothreitol (2 mM), Triton X-100 (0.015%), poly-A or poly-C (0.16 A_{260} units/ml), oligo-dT or oligo-dG (0.02 A_{260} units/ml), [^{3}H]-dTTp or [^{3}H]-dGTP (20 μM and 300 Ci/mol) and Carbopol as indicated. At appropriate times, carrier yeast RNA was added and DNA synthesis was determined as described above.

3. Results and discussion

The effect of Carbopol on the poly-A–oligo-dT dependent incorporation of dTMP residues catalyzed by R-MuLV reverse transcriptase is illustrated in fig.1. The severe inhibition by Carbopol is in sharp contrast with the eight-fold stimulation at high concentrations of Carbopol (160 μg/ml) observed by De Clercq and Claes [1] when they tested the endogenous reverse transcriptase reaction in viral core particles of M-MuLV.

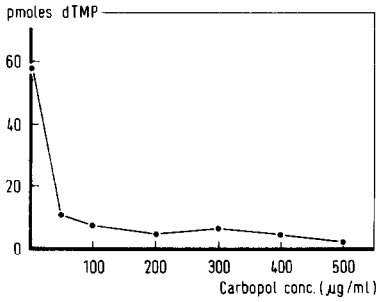

Fig.1. The inhibition of reverse transcriptase of R-MuLV by Carbopol using poly-A as a template. Samples were incubated during 60 min. In a parallel experiment (not shown) it was established that the incorporation was linear during the first 90 min of incubation and directly proportional to the amount of enzyme used. Other details were as described in section 2.2.

Since our result indicates that reverse transcriptases of different sources behave differently in interaction with Carbopol the effect on AMV-reverse transcriptase was tested, using poly-A as well as poly-C as templates (fig.2). Strong inhibitions were again observed at 100 μg/ml and higher concentrations of Carbopol.

Fig.3 illustrates that the inhibition by Carbopol is not confined to the transcription of synthetic templates.

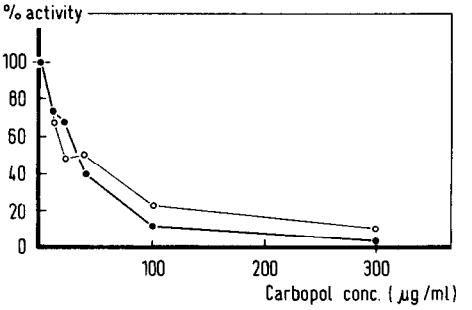

Fig.2. The inhibition of reverse transcriptase of AMV by Carbopol. Samples were incubated during 60 min. In a parallel experiment (not shown) it was established that the incorporation was linear during the first 120 min of incubation and directly proportional to the amount of enzyme used. (•) poly-A–oligo-dT dependent incorporation of dTMP residues (100% = 21.0 pmol). (○) poly-C–oligo-dG dependent incorporation of dGMP residues (100% = 12.2 pmol). The same amount of enzyme was used to test both activities. A lower template activity of poly-C has been reported before for this enzyme [9]. Other details see section 2.2.

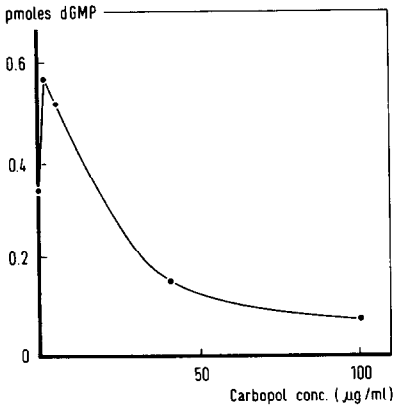

Fig.3. The effect of Carbopol on the endogenous activity of R-MuLV reverse transcriptase. Samples were incubated during 60 min. It was checked in a parallel experiment (not shown) that the incorporation was linear during the first 90 min of incubation both in the absence and in the presence of Carbopol (5 μg/ml and 40 μg/ml). Other details seen section 2.2

In this experiment the endogenous reverse transcriptase activity of R-MuLV cores was determined as a function of the Carbopol concentration. At very low concentrations only (1 and 5 μg/ml) a small stimulation was observed. Although not illustrated by the particular experiment depicted in fig.1, such stimulations were never found with synthetic templates. When the enzymatic activity was plotted as a function of the concentration of the poly-A–oligo-dT complex, it was found that Carbopol causes an inhibition of the competitive type (fig.4). This experiment was

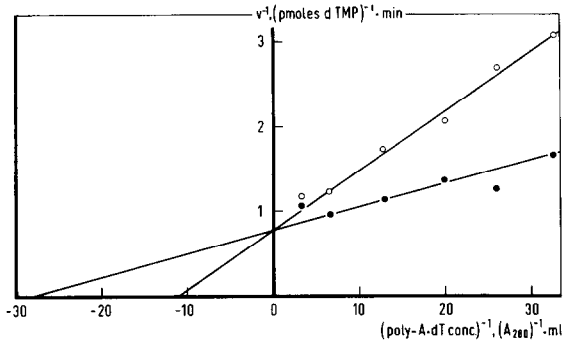

Fig.4. Lineweaver-Burk plot of reverse transcriptase of AMV. The reciprocal initial reaction velocity is plotted against the reciprocal concentration of poly-A–oligo-dT. The A_{260} ratio of poly-A and oligo-dT was 1 : 0.075 in all samples. (●) no Carbopol. (○) 10 μg/ml Carbopol.

carried out with the purified AMV-reverse transcriptase preparation. A similar experiment (not shown) carried out with viral particles of R-MuLV gave a mixed type of inhibition. The latter result may be due to the presence of other enzymatic activities in the virions. The observed competitive inhibition seems a logical result, because a negatively charged polymer like Carbopol could be expected to mimic nucleic acids and, thus, to interfere with the binding of the template to the enzyme. This result, however, is in contrast with

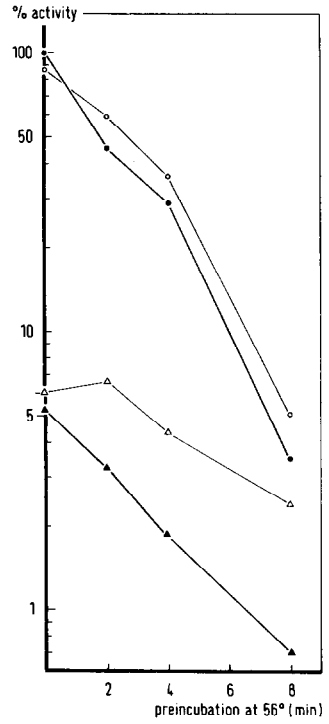

Fig.5. The effect of Carbopol on the heat denaturation of R-MuLV reverse transcriptase. The poly-A–oligo-dT dependent incorporation of dTMP residues was tested after a preincubation at 56°C, and percent activity (logarithmic scale) is plotted against the time of preincubation. Preincubation samples were rapidly cooled and assayed in the usual test at 37°C during 60 min. In all samples the same amount of one enzyme preparation was used. 100% activity represents 28.5 pmol of DTMP residues. (▲) One vol of Carbopol (40 μg/ml in saline) was added to the enzyme samples *after* preincubation at 56°C. As a result 3 μg/ml of Carbopol was present in the enzyme assay mixture. (△) One vol of Carbopol (40 μg/ml in saline) was added to the enzyme samples *before* the preincubation at 56°C. (●) One vol of saline was added to the enzyme samples *after* the preincubation at 56°C. (○) One vol of saline was added *before* the preincubation at 56°C.

the non-competitive inhibition of AMV-reverse transcriptase by pyran copolymers described by Papas et al [2]. An indication that Carbopol interacts directly with the enzyme is shown in the experiment depicted in fig.5, in which the heat inactivation of reverse transcriptase is studied in the presence and in the absence of Carbopol. In this experiment, enzyme samples were mixed with equal volumes of Carbopol (40 μg/ml in saline) or of saline alone, either before or after a preincubation at 56° C. When Carbopol was added *before* the preincubation at 56°C the enzyme was, of course, inhibited because of the presence of Carbopol in the assay system, but the enzyme was clearly better protected against heat denaturation than the enzyme samples which were heated in the absence of Carbopol. The solute (saline), on the other hand, had no effect on the heat denaturation. The final concentration of Carbopol in the assay system was only 3 μg/ml. Nevertheless, a very strong inhibition was observed (compare points at 0 min 56° C in fig.5), apparently due to the fact that Carbopol in a concentration of 20 μg/ml was present in the enzyme samples prior to their addition to the assay mixture. In the other experiments shown here, Carbopol was included in the assay mixture before the enzyme preparations were added.

There is presently a worldwide search for inhibitors of reverse transcriptase, because it is hoped that they are of potential value in the treatment of cancer [10]. However, reverse transcriptase activity is not required for the maintenance of the transformed state of cells, nor for mitosis of transformed cells [11]. Therefore, if effects of Carbopol on the immune system can be excluded the compound may be useful in discriminating between two causes of tumor growth: viral infection of uninfected cells and cell division of persistently infected cells.

Acknowledgements

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References

[1] De Clercq, E. and Claes, P. J. (1973) Biochim. Biophys. Acta 331, 328–332.
[2] Papas, T. S., Pry, T. W. and Chirigos, M. A. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 367–370.
[3] Braun, W., Regelson, W., Yajima, Y. and Ishizuka, M. (1970) Proc. Soc. Exp. Biol. Med. 133, 171–175.
[4] Regelson, W. (1967) Advan. Exp. Med. Biol. 1, 315–332.
[5] Chirigos, M. A. (1970) Comparative Leukemia Research, Bibl. Haemat. 36, 278–292.
[6] Chirigos, M. A., Turner, W., Pearson, J. and Griffin, W. (1969) Int. J. Cancer 4, 267–278.
[7] Verma, I. M. and Baltimore, D. (1974) Methods in Enzymology XXIX, pp. 125–143. Academic Press, New York, London.
[8] Chopra, H. C. and Shibley, G. P. (1967) J. Nat. Cancer Inst. 39, 241–256.
[9] Baltimore, D. and Smoler, D. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 1507–1511.
[10] Maugh, T. H. (1974) Science 184, 970–974.
[11] Linial, M. and Mason, W. S. (1973) Virology 53, 258–273.