INHIBITION OF RNA SLOW VIRUSES BY THIOSEMICARBAZONES

ASHLEY T. HAASE and WARREN LEVINSON, Veterans Administration Hospital and University of California Departments of Medicine and Microbiology, San Francisco, California 94121.

Received February 26, 1973

SUMMARY

N-methyl isatin β- thiosemicarbazone (Methisazone) effects comparable reductions in the reverse transcriptase activity and the cytopathic effect of the RNA slow viruses, Visna, Maedi and Progressive Pneumonia Virus. This is consistent with a requirement for RNA-dependent DNA synthesis in a lytic (non-transforming) cycle of virus growth.

In the 1950's Sigurdsson formulated the concept of slow virus infection based on his investigations of three diseases of Icelandic sheep, Visna, Maedi, and Rida, a variant of scrapie.[1] In each case transmissible agents were demonstrated that had the unusual property of producing disease only after a long incubation period, often measured in years. More recently, Gajdusek and his collaborators have shown that slow viruses are responsible for two chronic encephalopathies of man, Kuru and Creuzfeldt-Jakob disease, raising the possibility that other chronic degenerative afflictions of man may be due to slow viruses.[2]

Visna and Maedi are slowly progressive neurologic and pulmonary diseases respectively and the responsible agents have been shown to be closely related antigenically.[3] Recently the etiological agent of a chronic pneumonitis in Montana sheep, progressive pneumonia virus (PPV), has been shown to be a virus closely related to Visna and Maedi.[4] This small group of serologically related slow viruses share a number of characteristics with RNA tumor viruses including morphology, maturation by budding from cell surfaces,[5] inhibition of growth by inhibitors of DNA synthesis,[6] and 60-70 S RNA.[7] Of considerable current interest is the finding that the RNA slow viruses, like RNA tumor viruses, have an RNA dependent DNA polymerase (reverse trans-

criptase).[8] In contrast to RNA tumor viruses, however, the RNA slow viruses do not cause cancer in their natural host and interact with ovine cells in vitro in a lytic fashion causing syncytium formation, rather than cell transformation as is the case with RNA tumor viruses. The reverse transcriptase activity of the RNA tumor viruses is a necessary requirement for cell transformation since a mutant of an RNA tumor virus lacking the enzyme cannot transform cells.[9] The function of the reverse transcriptase in the non-transforming lytic cycle of growth of the RNA slow viruses is unknown at present, although the previously cited inhibition of growth with inhibitors of DNA synthesis suggests that RNA slow viruses may require this enzymatic function in a lytic cycle of growth.

We have found that N-methyl isatin β-thiosemicarbazone (M-IBT) inactivates in vitro (that is, on contact) the ability of avian, murine and feline RNA tumor viruses to replicate in and to transform cells (10 and unpublished observation). Recently we have determined that this compound and its analogue, thiosemicarbazone inhibit the reverse transcriptase activity of Rous sarcoma virus, an observation consistent with the requirement for this enzymatic function in transformation.[11] The correlation between inhibition of the enzyme and biological activity of the virus is similar to results obtained with rifampin derivatives.[12] These compounds, therefore, offered an opportunity to explore the function of the reverse transcriptase in RNA slow virus replication as well as the possibility that slow virus infection could be controlled by a chemotherapeutic agent, M-IBT, that is currently being used in man.[13]

METHODS

Visna and Maedi viruses were obtained from H. Thormar, New York Institute for Research on Mental Retardation, Staten Island, New York. Progressive Pneumonia Virus was from Dr. W. Hadlow, Rocky Mountain Laboratory, Hamilton, Montana. Viruses were propagated in sheep choroid plexus cells[14] and infectivity was assayed in sheep choroid plexus monolayers either by endpoint

determination of viral cytopathic effect or by plaque assay. In the endpoint determination, inocula of 0.1 ml of 0.5$\log_{10}$ dilutions of virus in L15 medium containing 2 percent heat inactivated lamb serum were added to confluent monolayers of cells in microtiter trays. Periodic assessments of cytopathic effect were made by light microscopy with final determination at 18 days. Titers were calculated by the method of Reed and Muench[15]. Plaque assays were performed on choroid plexus monolayers in 6 cm. plastic petri dishes. Inocula of 0.5 ml made up as described above were absorbed for three hours and thereafter 5 ml of overlay medium added that contained L15, 5 percent lamb serum, antibiotics, and 0.3 percent agarose. Plaques were visualized either by addition of neutral red to a final concentration of .003 percent or by fixation in formalin followed by staining of the cell sheet with 1 percent crystal violet in 20 percent ethanol. Large plaques (average diameter 3.4mm) were easily enumerated at two weeks and counts were constant at this time.

Viruses were purified from tissue culture medium of infected cultures with 50 percent saturated ammonium sulfate followed by centrifugation (SW 27 rotor, 22,500 RPM for two hours) through 17 percent sucrose on to a cushion of 40 percent (w/v) potassium tartrate. The virus band at the interface was then isopycnically rebanded twice in density gradients of 20 to 60 percent sucrose (w/v) (18 hours, SW 27 rotor at 25,000 RPM).

N-methyl isatin β-thiosemicarbazone (M-IBT) and its analogues, thiosemicarbazone (TSC), semicarbazide (SC), and methyl isatin (MI) were diluted in each experiment to a final concentration of 40μM. This series of compounds was chosen because TSC and M-IBT had been shown to be active against RSV, while SC and MI were inactive.[10] M-IBT and MI were obtained from K+K Rare Chemicals, Plainview, New York; SC and TSC were from Eastman Kodak. Experiments to test the effect of thiosemicarbazones on both virion reverse transcriptase activity and infectivity under lytic conditions were performed as follows Purified virus at a concentration of 5 x 10^7 PFU/ml. was diluted 1:20, ali-

TABLE 1

EFFECT OF THIOSEMICARBAZONES ON REVERSE TRANSCRIPTASE ACTIVITY AND INFECTIVITY OF VISNA VIRUS

	Enzyme Activity		Infectivity	
Treatment	(CPM)	% Reduction	(PFU)/ml.	% Reduction
DMSO	2257	---	4.8×10^7	---
M-IBT	339	85	7.4×10^6	85
TSC	288	87	3.0×10^6	94
SC	1710	24	3.6×10^7	25
MI	2323	0	4.4×10^7	8

In the infectivity assay, purified virus was diluted 1:20 in PBS and exposed to the various compounds (40 μM) or 1 percent DMSO as a control. After incubation at 37°C for 30 minutes, dilutions were made and residual virus quantitated by plaque assay. No toxic effect of drug on cells was seen. For enzyme assays, virus dilutions (1:20) were made into a reaction mixture which contained 10^{-1}M tris pH 8.1, 10^{-2}M $MgCl_2$, 10^{-4} dNTP's (dCTP, dATP, dGTP), and incubated for 30 minutes in the presence of various compounds. The virus was dissociated with NP 40 (final concentration 0.05 percent) and 5 μCi ^{3}H-TTP (specific activity 13.4 Ci/mM) were added. The mixture was incubated a further 60 minutes at 37°C and the incorporation of ^{3}H-TTP into acid insoluble CPM determined.

quots of 0.2 ml. dispensed into tubes, and 2 μl. of the various compounds added. After incubation at 37°C for 30 minutes, dilutions were made in tissue culture medium for infectivity assays. For assay of reverse transcriptase activity NP 40 detergent and ^{3}H-TTP were added, and, after an additional one-hour incubation at 37°C, acid precipitable counts/min. determined.[16]

RESULTS AND DISCUSSION:

A representative experiment using Visna Virus to show the correlation of inhibition of enzymatic activity and of infectivity by thiosemicarbazones is shown in Table 1. Both M-IBT and TSC reduced infectivity and reverse transcriptase activity comparably, by approximately 90 percent. In contrast, semicarbazide and methyl isatin lacked significant inhibitory activity (< 0.5 $\log_{10}$

reductions). The correlation between the loss of reverse transcriptase activity and infectivity was seen reproducibly in three separate experiments using both the plaque assay and the 50 percent end point cytopathic effect assay. Further, nearly identical reductions of 85 percent of virus infectivity occurred with M-IBT and TSC tested against Maedi and against PPV (data not shown).

The reduction of the Visna reverse transcriptase activity and coincident loss of the capacity of the virus to lytically and productively infect cells suggests that the RNA slow viruses may replicate in much the same way as RNA tumor viruses despite the difference in outcome for the cell. It is, however, possible that these compounds affect replication in some other way. For example, M-IBT, although it will not inactivate vaccinia virus on contact,[9] will inhibit vaccinia virus growth intracellularly by interfering with viral mRNA translation.[17] Whatever the mechanism of inhibition is, we have shown the possibility of controlling infection of three slow viruses by chemotherapy. This situation is particularly encouraging for in vivo work because of the relative lack of toxicity of thiosemicarbazones. For example, M-IBT has been used in man for smallpox prophylaxis and is being used in the treatment of smallpox vaccination complications.[18] Efforts to explore the efficacy of these drugs in vivo are in progress. Finally, the similarity of the results with RNA slow viruses to those obtained with RNA tumor viruses illustrate the fundamental similarity between persistent viral infections, whether the outcome ultimately is cell transformation and proliferation or cell dysfunction ending in death.

ACKNOWLEDGEMENTS:

This work was supported in part by VA Research funds and by USPHS Grants AI 08864, CA 12705, AI 06862, AI 08293, AI 00299, contract 71-2147 from the Special Virus Cancer Program of the National Cancer Institute, and Grant VC 70 from the American Cancer Society.

1. B. Sigurdsson, Brit. Vet. J. 110, 341 (1954).

2. D. C. Gajdusek, M. Alpers, Science 155, 212 (1967). C. J. Gibbs, Jr., D. C. Gajdusek, Science 165, 1023 (1969).

3. H. Thormar, Res. Vet. Sci. 6, 456 (1965).

4. K. Takemoto, et al. J. Virol. 7, 301 (1971).

5. H. Thormar, J. Cruickshank, Virology 25, (1965).

6. H. Thormar, Virology 26, 36 (1965).

7. F. Lin, H. Thormar, J. Virol. 7, 582 (1971). D. Harter, J. Schlom, S. Spiegelman, Biochem. Biophys. Acta. 240, 435 (1971). L. Stone, K. Takemoto, M. Martin, J. Virol. 8, 573 (1971) and F. Lin and H. Thormar, J. Virol. 10, 228, (1972).

8. F. Lin, H. Thormar, J. Virol. 6, 70 (1970). J. Schlom, D. Harter, S. Spiegelman, PNAS 68, 182 (1971). Stone, et al, Nature New Biology, 229, 257 (1971). L. Stone, K. Takemoto, M. Martin, J. Virol. 8, 573 (1971) and F. Lin and H. Thormar, J. Virol. 10, 228, (1972).

9. H. Hanafusa, et al.. Science 177, 1188 (1972)

10. W. Levinson, B. Woodson, J. Jackson, Nature New Biology 232, 116 (1971).

11. W. Levinson, A. Faras, B. Woodson, J. Jackson and J. Bishop, PNAS, 70, 164, (1973)

12. R. Ting, S. Yang, R. Gallo, Nature New Biology 236, 163 (1972).

13. D. Bauer, Ann. N. Y. Acad. Sci. 130, 110 (1965).

14. B. Sigurdsson, H. Thormar, P. Palsson, Arch. Ges. Virusforsch. 10, 365 (1960).

15. L. Reed, H. Muench. Am. J. Hygiene 27, 93 (1938).

16. A. Garapin, L. Fanshier, J. Leong, J. Jackson, W. Levinson, J. Bishop. J. Virol. 7, 227, (1971).

17. B. Woodson, W. Joklik, PNAS 54, 946 (1965).

18. R. Douglas, E. Lynch, M. Spira, Arch. Int. Med. 129, 980 (1972).