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(11)

TRANSLATION NO. 964

DATE: Dec 1963

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Fort Detrick
Frederick, Maryland

AN INFECTIOUS SUBTYPE OF THE NATURE OF RIBONUCLEIC ACID FROM BRAINS OF MICE INFECTED WITH YELLOW FEVER VIRUS

(Received 22 May 1962)

[Following is a translation of an article by G. Neilsen and J. Marquardt, of the Bernhard-Nocht-Institut für Schiffsund Tropenkrankheiten (Bernhard Nocht Institute for Ship and Tropical Diseases), Hamburg (Director: Dr. E. G. Nauck), Abteilung für Virusforschung (Virus Research Section) (Section Head: Dr. D. Peters), dedicated to Dr. E. G. Nauck on occasion of his 65th birthday, in the German-language periodical Archiv für die Gesamte Virusforschung (Archives for General Virus Research), Vol XII, No 3, 1962, pages 335-345.]

During the past year, phenol extraction, described by Gierer and Schramm (14) [numbers in parentheses refer to items in the appended bibliography], repeatedly proved its value in isolating infectious nucleic acids from material containing virus. The first effective preparation from a representative of the arthropod-borne virus group B was reported by Colter and others (10) for West Nile virus. Further results were then made known in rapid sequence for Murray Valley fever (1, 5), Japanese B encephalitis (22, 23), dengue (2, 22) and tick encephalitis (25, 26).

It had become evident in morphological studies of cell infection with yellow fever virus that the formation of intracytoplasmic areas containing ribonucleic acid occurs; the virus specificity of these areas is detectable with the immunofluorescent test. In the present study, a report is

made on the isolation of an infectious subtype of an RNA nature from a raw suspension of yellow fever virus by means of phenol extraction.

PROCEDURE

a. Virus

We used for the present studies a highly neurotropicly adapted yellow fever virus strain (Asibi neurotropic, Amsterdam Tropical Institute). The virus was continued on suckling mice from its 363rd intracerebral passage in young mice. The 15th and 16th passages were the original material for all the experiments.

Two to four day-old suckling mice were infected intracerebrally with 10^3 to 10^4 LD_{50}. After appearance of the disease symptoms the animals were killed by decapitation, their brains were removed sterilely and poured into flasks as a 50% homogenization in Earle's buffer (pH 7.2) + 10% calf serum and were preserved at -70°C. until extraction.

b. Production of the Nucleic Acid Preparations

After thawing, a 10% brain suspension is produced by adding buffer; this suspension, after being homogenized in a Potter-Elvejhem apparatus, is centrifuged first at low speed (10 mins. at 3000 RPM) and finally at high speed (45 mins. at 10,000 RPM). The residue is shaken out three times with 80% aqueous phenol in imitation of Gierer and Schramm (14), first with an equal volume of phenol (8 mins.), then with half volume (5 mins.) and finally with one fourth of the volume (3 mins.) of phenol. Aqueous and phenol phases were separated by six minutes of centrifugation at 6,000 RPM. Phenol left over in the aqueous phase is removed by means of shaking out three times with abundant ether. After separation of the phases, the ether was driven out by introducing nitrogen. Work-up took place at 0° to 4° C.

c. Infectiosity Tests

Virus suspension and phenol extracts were inoculated intracerebrally in suckling mice after a $\log_{10}$ dilution. In order to equalize variations depending on the animal, all the experimental mice in one experiment were mixed specifically and eight suckling mice were returned arbitrarily to the mother animals. The inoculation dose amounted to 0.02 ml. per animal. For purposes of computation, animals that died

from four to 10 days after the infection were used exclusively. The infectiosity titers were computed with Kärber's method (17) with the aid of tables from Lorenz (20) [See note]. To equalize the dilution (10%) the initial concentrations of the phenol extracts were inserted in the computation as a 10% dilution.

([Note:] We thank Mathematics Diplomate [Dipl. math.] R. J. Lorenz (Federal Research Institute, Tübingen) for having been so kind as to give us a copy of the tables set up by him.)

d. Biochemical Determinations

RNA was determined with orcin in accordance with Ceriotti's (9) method, after first precipitating with alcohol. The detection of small amounts of protein occurred with the aid of the biuret test according to Schuster and others (24). The phenol extract was diluted with phosphate buffer 1:20 for UV spectrophotometric measurements and was measured in a Zeiss M4Q spectrophotometer in 1 cm. quartz cuvettes with an M/2 phosphate buffer (pH 7.1).

e. Enzymes

Trypsin inhibitor (2X crystallized from soy beans) and ovomucoid, Worthington.

Abbreviations: ribonucleic acid = RNA; deoxyribonucleic acid =DNA; ribonuclease = RNase; deoxyribonuclease = DNase.

RESULTS

Infectiosity of the Phenol Extracts

An infectious principle could normally be isolated from mouse brains infected with yellow fever by means of extraction with cold phenol. This principle differs in many respects from the infectious activity of the cerebral homogenization.

The phenol extracts contained, on the average, 0.8 to 1.0 mg of RNA per ml. Precipitation with ethanol was accomplished before determination, in order to produce an orcin reaction without turbulence and to eliminate smaller RNA fragments. The ratio of absorption at 280 mμ to

absorption at 260 mμ was 1:2.02 in the UV spectrophotometric measurement. The biuret test made according to Schuster and others (24) came out negative; therefore, the protein content of the extracts was under 50 γ per ml. 0.02 ml of the phenol extracts contained 2.7 X 10^2 to 1.5 X 10^3 LD_{50}, which corresponds to about 1/30,000 of the original infectiosity (Table 1). With respect to equal infectiosity doses of phenol extract and original virus, there was extreme agreement in the time course of the disease symptoms and the death rates. In a neutralization experiment in the presence of yellow fever immune serum [See Note], infectious cerebral homogenizations from phenol extract and original virus passages behaved in the same way.

([Note:] We are much obliged to Dr. Schindler, Hamburg Tropical Institutes, for having given us yellow fever immune monkey serum.)

TABLE 1. Infectiosity of Original Virus and RNA Fraction

	LD_{50} (log) per 0.02 ml						
Experiment number	103	104	109	112	114	115	118
Original Virus	6.84	7.45	7.50	7.35	7.36	7.50	7.38
RNA fraction	2.75	2.50	3.17	2.17	2.50	2.43	3.13

The finding that after treatment with phenol only about 0.03% of the original infectiosity can be recovered, requires a further characterization of the infectious agent in comparison with the original virus. It is known from earlier studies (3, 4, 26) that the behavior of infectious phenol extracts exhibits fundamental differences, in comparison with the untreated virus, in the presence of enzymes, alcohol, temperature effects, etc.

Differentiation by Means of Nucleases

Phenol extracts and cerebral homogenizations were mixed with antibiotics, were regulated in equal volumes to 100 tc 300 LD_{50} per 0.02 ml and incubated at 0°C. after the addition of 0.1 vol. of enzyme solution. This suboptimal temperature for enzymatic action was chosen in order to avoid thermal damage to the phenol extracts.

Table 2 shows the results of treatment with DNase and RNase. The death rate of mice that had been infected

with virus suspensions treated with enzymes remains unchanged in comparison with control experiments. In contrast with this, the infectiosity of the phenol extract can be increased already with 0.01 γ per ml of RNase. The phenol extracts proved to be very stabile, on the other hand, with DNase treatment (in the presence of 0.003 mol $MgSO_4$).

TABLE 2. Infectiosity of Original Virus and RNA Fraction Before and After Treatment with Nucleases

Experiment	LD_{50} (log) per 0.02 ml					
	Original Virus			RNA Fraction		
	Original	+RNase*	+DNase**	Original	+RNase***	+Dnase**
103	6.84	7.40	7.35	2.75	0	2.50
104	7.45	7.50	--	2.50	0	--
114	7.63	7.63	7.50	2.50	0	2.46
115	7.50	7.43	7.55	2.43	0	2.60

* 10 to 100 γ per ml of RNase, 0° C., 10 to 90 mins.
** 1 to 10 γ per ml of DNase, 0°C., 90 mins.
*** 0.01 to 10 γ per ml of RNase, 0° C., 5-60 mins.

In conformity with the procedures outlined above, specifically equal infectiosity doses (100 to 300 LD_{50} per 0.02 ml) of the phenol extract were mixed with graduated RNase concentrations and the effect was examined at incubation times of various lengths, in order to determine the minimal effective dose of enzymes. A specific litter of mice was used for each concentration and time. The effect was indicated by the proportion of the number of dead mice to mice used.

The effect of this series of studies of graduated enzyme concentrations and incubation times is shown in Table 3 (columns 1 and 2). In spite of the high admixture of cell RNA, the phenol extracts are already inactivated within 5 to 10 minutes with 0.01 γ per ml of enzyme.

Effect of Proteases

Studies directed toward the action of trypsin had shown unexpectedly that this enzyme also makes it possible to increase the infectiosity of the phenol extracts, but not of the intact virus. In order to arrive at an explanation of the way in which it acts (possible RNase contamination), a series of experiments were undertaken to determine the minimal dose of the enzyme. Table 3 (columns 3 to 6) indicates that 1.0 γ per ml of trypsin increases the infectiosity of the phenol extract within 5 minutes, whereas chymotrypsin remains without effect on phenol extract and original virus, even with a higher concentration.

TABLE 3. Determination of the Smallest Effective Concentrations of RNase and Trypsin

		RNase	RNase	Trypsin	Trypsin	Trypsin	Chymotrypsin
LiBr, 0.02 mol	Original	7.45	7.50	7.35	7.45	7.50	7.38
	Original + enzyme	7.50 (10 γ/ml 60 Min).	—	—	7.50	7.45 (10 γ/ml 60 Min).	7.63 (10 γ/ml 60 Min).
	Phenol extract	2.50	3.17	3.17	2.50	3.17	3.13
Phenol extract + enzyme							
0.001 γ/ml	5 Min.	—	—	—	—	—	—
	15 Min.	8/8	8/8	—	—	—	—
	60 Min.	8/8	8/8	—	—	—	—
0.01 γ/ml	5 Min.	—	6/8	—	—	8/8	—
	15 Min.	8/8	0/8	—	8/8	8/8	—
	60 Min.	0/8	—	—	8/8	8/8	—
0.1 γ/ml	5 Min.	—	0/8	8/8	—	8/8	8/8
	15 Min.	0/8	0/8	8/8	8/8	8/8	8/8
	60 Min.	0/8	—	7/8	7/8	7/8	8/8
0.5 γ/ml	5 Min.	—	0/8	—	—	7/8	—
	15 Min.	0/8	0/8	—	—	1/8	—
	60 Min.	0/8	—	—	—	1/8	—
1.0 γ/ml	5 Min.	—	0/8	0/8	—	0/8	8/8
	15 Min.	0/8	0/8	0/8	0/8	0/8	8/8
	60 Min.	0/8	—	0/8	0/8	0/8	8/8
10 γ/ml	5 Min.	—	0/8	—	—	—	8/8
	15 Min.	0/8	0/8	—	0/8	—	8/8
	60 Min.	0/8	—	—	0/8	—	8/8

[In above columns, the proportion of the number of dead mice to mice used is given.]

According to M. Laskowski and M. Laskowski, Jr. (18), an equimolar addition of trypsin inhibitor or ovomucoid should quickly reduce the trypsin activity. Therefore, we attempted to gain further insights into the way in which trypsin acts by experimenting with an inhibited enzyme (Table 4). Even after increasing the inhibitor concentration we saw no difference in action in comparison with unblocked trypsin. Under the assumption that trypsin also enters into an inactive, reversible addition compound with the infectious RNA (15), we treated the enzyme-substrate complex again with phenol. The infectiosity, however, remained raised, while a control extraction did not impair the activity of the infectious RNA.

TABLE 4. Action of Trypsin and Trypsin Inhibitors on the RNA Fraction

Enzyme ml	Inhibitor	Action Time, mins.	Death Rate of Mice
Trypsin 1.0 γ	--	15	0/8
0.1 γ	--	15	8/8
--	Ovomucoid;	60	8/8
Trypsin 1.0 γ	4 γ p. ml, 60 mins.	60	0/8
--	Trypsin inhibitor;	30	8/8
Trypsin 1.0 γ	2 γ p. ml, 30 mins.	30	0/8
--	Trypsin inhibitor;	30	8/8
Trypsin 1.0 γ	10 γ p. ml, 30 mins.	30	0/8

RNA fraction test LD_{50} $10^{3,17}$ per 0.02 ml.

Action of Ethyl Alcohol

Two vol. of ethanol were added at 0° C. to the dilutions of yellow fever virus suspension and phenol extracts adjusted to the test range and this was centrifuged at low speed. The sediment was absorbed again in a equal volume of buffer and inoculated.

The results of precipitation with ethanol presented it Table 5 show differences in the behavior of a raw suspension of virus and a phenol extract. Even a 10,000 times

higher infecting dose in the virus suspension is inactivated by alcohol, while precipitation and putting in suspension again remained without perceptible reaction on the infectiosity.

Action of Temperature Effects

Preparations of raw suspensions of virus and of phenol extracts in equal doses are exposed to the temperature conditions shown in Table 6. Treatment lasting 30 mins. at +60°C. increases the infectiosity of virus and extract, while incubation for 60 mins. at 37° C. is tolerated. The phenol extract turns out to be thermostabile only at -70°C. within the fourteen day observation period.

TABLE 5. Behavior of Original Virus and RNA Fraction in the Presence of Ethanol

	LD_{50} (log per 0.02 ml.		
Experiment	109	114	116
Virus, untreated	7.50	7.63	7.87
Virus + ethanol	0	0	0
RNA fraction, untreated	3.17	2.57	2.67
RNA fraction + ethanol	2.85	2.38	2.45
After concentration	--	--	3.38

TABLE 6. Effect of Temperature on Original Virus and RNA Fraction

Experiment	Temperature Action °C.	LD_{50} (log) per 0.02 ml			
		Before Treatment		After Treatment	
		Virus	RNA Fraction	Virus	RNA Fraction
109/11	-70°;14 days	7.50	3.17	7.45	2.75
114	-20°;14 days	7.63	2.50	6.87	0
	-70°;14 days	7.63	2.50	7.65	2.38
115	+37°;60 mins.	7.50	2.43	7.28	2.45
	+60°;30 mins.	7.50	2.43	0	0
116/117	-70°;14 days	7.87	3.38	7.56	3.75

Action of Serum and Globulin

We found, in agreement with other authors (3, 4, 11, 21, 25, 26), that phenol extracts lose their infectiosity extraordinarily easily when they are mixed with serum. The RNase content of the serum (3, 4, 16) is held responsible for this reaction. Immune globulin preparations produced by precipitation are supposed to lose the inhibitory action of serum, while the virus suspensions remain completely neutralizable.

By adding an equal volume of saturated $(NH_4)_2SO_4$ solution to normal and hyperimmune monkey serum, the globulin component was precipitated. After washing out with a 50% $(NH_4)_2SO_4$ solution, we absorbed the sedimented precipitate in 2/3 of the original volume of phosphate buffered (M/100) physiological NaCl solution and dialyzed it with the same buffer until the disappearance of sulfates. For the neutralization tests, equal infectiosity doses (200 to 400 LD_{50} per 0.02 ml) of virus suspension or phenol extract were mixed in equal parts with precipitating globulin dilutions, were left for 30 minutes under refrigeration with occasional agitation and subsequently were inoculated.

While the infectiosity of the virus suspension was neutralizable exclusively by means of immune globulin, the phenol extracts were inhibited with normal and immune globulin. Dilution of the globulins (>1:128) reduced the inhibitory action. A repetition of the experiments dispensing with a particular action time of the globulins (mixing the reaction components in the injection syringe) confirmed the result.

DISCUSSION OF THE RESULTS

Previous studies (2, 5, 11, 22, 23, 25) had shown that the infectiosity of several viruses of the arthropod-borne group B is connected with a subtype of an RNA nature. It could be assumed from the fact that it belongs to these viruses and from earlier histo-chemical studies (7) that the yellow fever virus is to be classified under RNA viruses. It was possible to confirm this supposition by means of the successful production of an infectious RNase labile product after phenol extraction from raw suspensions of virus.

In agreement with other authors, we designate this fraction as "infectious ribonucleic acid." The problem of other substances found in the extract that are possibly significant for the action must remain open. The fraction turned out to be free of proteins when it was tested with the usual methods of analysis. The infectiosity (1/30,000 of the original virus) present in the phenol extract probably was not caused by an intact virus, since such a virus is enzyme-stabile in the presence of infectious RNA. The difference in titer that we observed between RNA and the original virus agrees with the results of other experiments. Colter and Ellem (12, 13) substantiate this fact that only a partial amount of the infectious RNA comes into action. The inactivation of releasable RNA by means of trypsin is unexpected and unexplained in its peculiarities. Bachrach (6) reported a similar finding for the RNA of the hoof and mouth disease virus. There is a further observation (15) on a DNA preparation from ϕX 174 phages. This effect probably cannot be attributed to a proteolytic action of trypsin, since the addition of inhibitors had no effect on the inactivating action of the enzyme. The fact that chymotrypsin does not inhibit RNA argues in favor of this opinion.

It can be inferred from the dose-action relationship of the enzymes indicated in Table 3 that the trypsin used

must have contained at least 2% of RNase as an impurity, and that an RNase action was probably responsible for the inactivation. We are not familiar with data that might support the assumption of such a high RNase contamination of highly purified trypsin. The role of trypsin in the inactivation of DNA phages was able to be explained by Guthrie and Sinsheimer (15). They found that nucleic acid and enzyme protein combine in an addition compound with loss of infectiosity. Until now there have been no studies of this nature for ribonucleic acids. The inactivating action of RNase-free proteins established by Colter and Ellem (10, 14, 15) in Mengo virus RNA could, however, probably let this unspecific enzyme type of reaction appear to be possible.

Intact virus and infectious RNA reacted differently to precipitation with ethanol. While the RNA was not affected by precipitation, the virus was entirely inactivated. There are agreeing results for the tick encephalitis virus obtained by Sokol and others (25, 26). They suspect that the inactivation occurs in the presence of the MM virus (19) due to the direct action of alcohol.

Yellow fever virus, like most of the arthropod-borne B viruses, is thermolabile. This may explain why we failed to discover greater differences in the temperature behavior of untreated virus and infectious RNA. The RNA remains stabile for several weeks only at -70° C. Similar findings were reported for Murray Valley Fever (4) and the tick encephalitis virus (26).

Neutralization studies on RNA preparations require the removal of the RNase serum. Alexander and others (3) obtained, by means of precipitation, globulins that had no more effect on infectious polio RNA. On the other hand, Mussgay and Strohmayer (21) established inhibitory actions of homologous and heterologous immune globulins in hoof and mouth disease RNA, just as Sokol and others (25) did for tick encephalitis RNA. In our experiments, the infectiosity of the RNA was increased, independently of the duration of the action, both with normal and immune globulin; intact virus was neutralizable only with immune globulin. The decision on whether the globulin action observed by us is the cause of the protein inhibition of infectious RNA described by Colter and Ellem (14, 15), must remain open, in view of the difficulty in dealing with the smallest RNase contaminations.

The present experiments were performed on little purified raw suspensions of virus. Further experiments will probably explain whether the infectious RNA results from the virus itself or from its vegetative first stages in the host cell.

SUMMARY

Ribonucleic acid, with a 0.03% infectiosity of the original virus, was obtained by phenol extraction from the brains of mice infected with yellow fever. The preparation displayed the UV absorption that is characteristic of nucleic acids. Protein admixtures were not detectable with the biuret test. Ribonuclease, trypsin and also serum globulin inhibited the infectiosity. Deoxyribonuclease, chymotrypsin and ethanol were without effect. The inhibitory action of trypsin is discussed.

We thank Dr. D. Peters for his helpful interest in our work and Miss U. Lehmann for her excellent technical assistance.

The present studies were conducted with the support of the Deutsche Forschungsgemeinschaft (German Research Society).

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