Antiviral Activity of Tobacco Leaf on Encephalomyocarditis (EMC) Virus Infection[1] (35990)

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Through our antiviral screening work in the field of natural products of higher plant origin for neurotropic RNA virus infections at animal level (1, 2), a water-soluble and ethanol-insoluble fraction of tobacco leaf extract has been found to have antiviral activity against encephalomyocarditis (EMC) virus infection in mice. The extract also showed antilethal activity on lymphocytic choriomeningitis (LCM), a delayed type of hypersensitivity induced by LCM virus infection. This paper reports chiefly the anti-EMC activity of tobacco leaf both *in vivo* and *in vitro* in comparison with poly I:C (3) and tilorone hydrochloride (4, 5) as standard antiviral (interferon inducing or releasing) agents.

Materials and Methods. 1. In vivo *test.* Agents were injected subcutaneously (sc) or intraperitoneally (ip) 20 or 4 hr before, or 3, 24, or 48 hr after sc or ip inoculation of LD_{70-90} of EMC virus. Both single and repeated doses were administered. Swiss albino mice weighing about 17 g were used.

2. In vitro *test.* Agents were added to the medium of KB cell cultures at the time of virus inoculation. The viruses used were EMC, polio type 2, vesicular stomatitis (VS), reo type 2, vaccinia IHD strain and adeno type 12 which had been passaged at least several times in KB cells before use. The medium (medium 199 with 2% calf serum) containing antiviral agents was changed every 2 days. The amount of virus and the degree of cytopathic effect (CPE) in treated cultures were recorded when they reached maximum in the control cultures.

3. Titration of virus in brain. One infected brain was ground with 5 ml of Hanks' solution, and the supernatant after centrifugation (5000 rpm, 20 min) was designated as 4×10^{-1} dilution, the starting concentration for virus titration. Each 10-fold dilution was inoculated into 3 mice intracerebrally (ic), allowing calculation of LD_{50}.

4. Titration of interferon in serum. The cardiac blood was collected 5 or 20 hr after a single ip injection of agents, diluted 4 times with medium 199, centrifuged; and the serum was designated as the 1:4 dilution. This was the starting concentration for interferon assay. Cultures of L cells in tubes (1 ml) were incubated with the 0.1 ml of serial 4-fold dilutions of serum for 24 hr. After removal of the serum by 3 rinses with Hanks' solution, the cells were challenged with 100 $TCID_{100}$ of vesicular stomatitis virus and reincubated for 2 days. Interferon titers were expressed as the reciprocal of the maximum dilution showing 50% CPE reduction. The L cells were maintained in Eagle's MEM with 5% calf serum.

5. Preparation of tobacco extract. Sixteen grams of tobacco leaf (20 cigarettes of Parliament brand, freed from paper) was extracted in 2000 ml of hot water (60 to 80°) for 1 hr. The concentrated water extract, after flash-evaporation to 100 ml and centrifugation, was dropped into 1000 ml of 95% ethanol; and the precipitate was collected and dried after 3 times washing with ethanol. About 2 to 3 g of brown powdery solid containing 9% polysaccharide and no alkaloids was obtained (named NT-W-EP). For further fractionation, 40 ml of water solution of NT-W-EP at concentration of 50 mg/ml was passed through 1 g of carboxymethylcellulose (CM) and then 1 g of diethylaminoethyl cellulose (DEAE) columns (designated NT-CM-DEAE), then ultrafiltered through

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TABLE I. Effect of Multiple Administration of Tobacco Extracts in Mice after EMC Infection; Comparison with Poly I:C.

Agent	Dose (mg)	Treatment (sc) Started (hr)	Continued	No. died/total Treated	Control	Treated/control (%)	p
NT-W-EP	3[a]	+3	2/d/5 days[b]	19/50	41/50	46	<.001
	3	+3	2/d/5 days	4/10	9/10	44	<.05
	3	+24	2/d/4 days	4/10	9/10	44	<.05
	3	+48	2/d/3 days	8/10	9/10	89	.4
	3	+3	2/d/5 days	4/10	8/10	50	.07
	1.5	+3	2/d/5 days	5/10	8/10	63	.15
	0.8	+3	2/d/5 days	8/10	8/10	100	
	(μg)						
Poly I:C	20	+3	2/d/5 days	9/20	18/20	50	<.005
	20	+24	2/d/4 days	12/20	18/20	67	<.005
	20	+48	2/d/3 days	18/20	18/20	100	

[a] Maximum nontoxic dose (MNTD) for this schedule.

[b] Treatment (sc, twice daily for 5 days) started 3 hr after ip infection; young adult Swiss albino mice.

UM-10 membrane (Amicon) at 50 lb of nitrogen pressure. The retentate (designated NT-R: dark brown) was diluted to the original volume and contained 10 mg/ml of brown solid; the filtrate (designated NT-F; slightly yellow) contained 20 mg/ml of white solid.

6. Poly I:C and tilorone HCl. Poly I:C (1 mg/ml solution) was purchased from Microbiological Associates and tilorone HCl was kindly supplied by the Wm. S. Merrell Company.

Results. 1. Antiviral activity of tobacco extract on EMC virus infection in mice. The initial fraction (NT-W-EP) of tobacco extract was tested against EMC virus infection in mice by using the established screening methods which have been used for 10 years in this laboratory: the results are comparable with our previous data (1, 2). Table I shows the comparison with poly I:C. It was found that the crude fraction decreased the mortality of EMC infection to one half of control deaths when twice daily treatment was started 3 hr after inoculation of the virus. It was still active when the start was delayed for 24 hr, as in the case with poly I:C, which is one of the most active agents against EMC infection we have tested. Then, to confirm that the prevention of the fatal paralysis was due to the inhibitory effect of the agent on growth of EMC virus in brain, the amount of virus in the individual brains of mice 5 days after infection was titrated. Table II shows the results. The schedule of the treatment was the same as before. Although there was much variation in the virus titers in the treated group, there was a significant inhibition of virus growth (av 3.6 log reduction) in comparison with the control group. Thus, tobacco agent suppressed the propagation of EMC virus in mouse brain when the virus was inoculated ip.

2. Prophylactic effect of tobacco extract on EMC virus infection. Because poly I:C and

TABLE II. Effect of Tobacco Extracts on Growth of EMC Virus in Brain of Mice.[a]

Brains taken 5 days after infection ip	Amount of infective virus (10 log LD_{50}/0.03 ml) Treated	Control
Brain no. 1	4.5	7.5
2	2.8	6.5
3	0.5	7.2
4	4.2	5.2
5	3.2	6.8

[a] Treatment with NT-W-EP (3 mg, sc) started 3 hr after ip inoculation of LD_{70-90} of EMC virus; given sc twice daily for 5 days. Clinical symptoms became evident on day 5, the virus titer in controls approached maximum.

TABLE III. Prophylactic Effect of Tobacco Extracts on EMC Virus Infection in Mice: Comparison with Poly I:C and Tilorone.

Agent	Dose	Pretreatment (ip)[a] (hr) First	Pretreatment (ip)[a] (hr) Second	No. treated/total Treated	No. treated/total Control	Treated/control (%)	p
NT-W-EP	3 mg	−24	−4	14/30	28/30	50	<.001
NT-CM-DEAE	3 mg	−24	−4	12/40	35/40	39	<.001
	3 mg	−24	None	8/30	24/30	33	<.001
NT-F	2 mg	−24	None	24/50	43/50	56	<.001
	2 mg	None	−4	28/70	56/70	50	<.001
NT-R	2 mg	−24	None	13/50	42/50	31	<.001
	2 mg	None	−4	11/40	29/40	38	<.001
Poly I:C	20 μg	−24	−4	4/20	18/20	22	<.001
	20 μg	−24	None	12/40	33/40	36	<.001
	20 μg	None	−4	6/20	18/20	33	<.05
Tilorone	1 mg	−24	None	3/10	9/10	33	<.05
	1 mg	None	−4	13/40	33/40	39	<.001
	5 mg drink	−24	None	6/20	18/20	33	<.001

[a] Treatment was given ip 24 hr and/or 4 hr before ip inoculation of LD_{70-90} (1000 TCID) of EMC virus.

tilorone have remarkable prophylactic activity (3–5), it was next determined whether the tobacco agent also has prophylactic activity. A single or double injection of tobacco extract ip 24 hr and/or 4 hr before EMC ip inoculation was made. Table III shows the results. It shows that one injection, either 24 or 4 hr before virus inoculation, protected the mice from death. Fraction NT-R which could not be passed through UM-10 membrane (passes substances below 10,000 mol wt), poly I:C, and tilorone all appeared to be more active than Fraction NT-F, but the latter fraction was interesting because it seemed to have an active principle with a lower molecular weight, estimated at about 1000 by Sephadex G50 column. This appearance of prophylactic activity of the agents is related to the combination of infection and treatment routes. Table IV suggests that tobacco agent and tilorone were not effective when they were injected sc against ip virus inoculation, while poly I:C was active by this route. As already shown in Table I, the multiple administration of tobacco agent was needed for the appearance of activity by the sc route against the ip virus inoculation.

3. Interferon assay in serum of mice treated with tobacco extract. The results of the experiments shown in Table III and IV, suggested that tobacco agent might be an interferon inducer. Therefore, titration of serum interferon in the treated mice was done by using a system of VS virus and L cells. The amount of interferon in the serum of mice, collected 5 and 24 hr after a single ip injection of agents was measured. Unexpectedly, both NT-F (4 mg) and NT-R (4 mg) fractions of tobacco extract were found to have no activity (<4) to induce serum interferon, while poly I:C (0.1 mg) and tilorone (2 mg) induced definite amounts of interferon (1024 and 64 at 24 hr).

4. Antiviral activity of tobacco extract on EMC virus in KB cell cultures. To investigate the mode of antiviral action of tobacco agent, a system of EMC virus and KB cells, which has been used for 6 years and produces results comparable with our previous data (2), was adopted. Poly I:C and tilorone were also tested for comparison. The results show that only the low molecular fraction NT-F (1 mg/ml) suppressed the CPE and propagation of EMC virus in KB cells when agents were added at the time of infection and kept in the medium during the incubation period. Pretreatment with any agent for 20 hr before infection did not make the cells resistant to

TABLE IV. Effect of Routes of Treatment and Infection on Prophylactic Activity of Tobacco Extracts in Mice.[a]

Agent	Dose	Route of Infection	Route of Treatment at —4 hr	Died/total Treated	Died/total Control	Treated/control (%)	p
NT-F	2 mg	ip	sc	8/10	8/10	100	
NT-R	2 mg	ip	sc	8/10	9/10	89	.39
Poly I:C	20 μg	ip	sc	4/10	8/10	50	.07
Tilorone	1 mg	ip	sc	8/10	8/10	100	
NT-F	2 mg	sc	ip	9/30	25/30	36	<.001
NT-R	2 mg	sc	ip	7/20	16/20	44	<.005
Poly I:C	20 μg	sc	ip	3/10	8/10	38	<.005
Tilorone	1 mg	sc	ip	4/10	8/10	50	.07
NT-F	2 mg	sc	sc	5/20	18/20	28	<.001
NT-R	2 mg	sc	sc	5/20	18/20	28	<.001
Poly I:C	20 μg	sc	sc	2/10	10/10	20	<.001
Tilorone	1 mg	sc	sc	6/20	18/20	33	<.001

[a] A single treatment was made ip or sc 4 hr before ip or sc inoculation of LD_{70-90} of EMC virus.

the challenge of the virus. Our preliminary experiments indicated that even poly I:C (50 μg/ml) mixed with 100 μg of neomycin could not make the KB cells refractory to EMC virus, while the same combination made the L cells refractory. Therefore, the KB cells seem to be defective to interferon production by poly I:C or tilorone (2 μg/ml). Probably the antiviral activity of NT-F fraction of tobacco extract on EMC virus in KB cells is not due to an indirect action via the machinery of interferon induction, but is due to a direct action of the agent on the cells. This idea was supported by the fact that NT-F fraction was still effective against EMC virus reproduction even when it was added 2 or 3 hr after infection in the single-cycle growth experiment (2). Briefly, KB cells were infected with high multiplicity of EMC virus (100 $TCID_{100}$/cell). One hour later, the cells were washed 5 times with Hanks', then 4 mg of NT-F was added at various times postinfection and incubated for 15 hr. The CPE at 15 hr was recorded. The results show that during the first hour of incubation with the mixed virus and NT-F fraction, the adsorption of the virus onto the cells could not be interfered with by NT-F; however, the intracellular viral process was completely suppressed before the stage of appearance of CPE when NT-F was added to the medium within 2 hr after infection.

5. Antiviral activity of tobacco extract on several viruses in KB cells. The antiviral activity of NT-F fraction against several cytopathic viruses which have been passaged in KB cells in this laboratory was tested. As shown in Table V, it was found that the EMC and VS viruses were most sensitive, polio type 2, reo type 2, and vaccinia viruses were moderately sensitive, and adeno virus type 12 was resistant to the action of NT-F.

6. Antilethal activity of tobacco extract on LCM virus infection in mice. The crude fraction of tobacco extract (NT-W-EP) was also found to reduce the mortality of lymphocytic choriomeningitis (LCM) which is a delayed type of hypersensitivity induced by the intracerebral (ic) inoculation of LCM virus in mice (6). Table VI shows the results. In comparison with three standard imunosuppressive drugs, tobacco extract seems to have definite antilethal activity against the fatal paralysis. On the contrary, poly I:C was not effective against LCM infection.

Discussion. Tobacco leaf of cigarettes has been found to contain antiviral agents which decrease the mortality of EMC and LCM virus infections in mice. The anti-EMC activity, which is due to the suppression of virus growth in brain, was demonstrated after a single ip injection, but the mode of action

TABLE V. Antiviral Activity of Tobacco Extract on Several Viruses in KB Cell Cultures.[a]

Virus	Inoculum size ($TCID_{100}$)	Incubation period (day)	CPE	
			NT-F treated (2 mg/ml)	Control
EMC	1000	1	0	4+
	100,000	1	1+	4+
VS	10,000	1	0	4+
	100,000	1	1+	4+
Polio type 2	100	2	1+	4+
	1000	1	2+	4+
Reo type 2	10	6	2+	4+
Vaccinia	1000	6	2+	4+
Adeno type 12	10	6	4+	4+

[a] The agent was added to the culture medium at the time of virus inoculation. Medium with the agent was changed every 2 days. Incubation was stopped when CPE in controls reached maximum (4+).

seems to be somewhat different from that of interferon inducers such as poly I:C and tilorone HCl. The tobacco agents did not induce any detectable amount of interferon in the blood, although they made the mice resistant against EMC infection. Also, contrary to the negative anti-EMC activity of poly I:C and tilorone in KB cells, tobacco fraction (NT-F) suppressed virus reproduction in the cells even when it was added 2 hr after infection. Petreatment with NT-F for 20 hr did not make KB cells refractory to EMC infection. These data suggest that the mode of action is not due to interferon induction but to direct effect on intracellular viral processes. Another tobacco fraction (NT-R), active *in vivo,* has no antiviral activity in KB cells, so its mode of action is not clear. NT-R apparently does not enhance the immunological defense systems of mice; at least the fraction did not stimulate or suppress antibody production against sheep red cells in mice. However, Pindak *et al.* (7) reported that interferon titers in serum did not correlate with the degree on protection against MM virus (Columbia SK-EMC group) infection when statolon or poly I:C (high interferon inducers) were compared with *E. coli* endotoxin or pyran (low inducers, but affording good protection); therefore it cannot be excluded that tobacco agent may stimulate interferon, which is then bound in the cells. The anti-LCM activity of tobacco agent seems not to be due to antiviral action, because LCM virus propagation in brain has been found not to be inhibited in a recent experiment; this is in contrast to the anti-EMC action. The mode of action of tobacco agent on LCM disease might be an immunosuppression against the virus-induced, delayed type of hypersensitivity.

Tobacco extract, after removing alkaloids, can be separated into two active portions, NT-F and NT-R, by molecular filtration (UM-10 membrane: cut substances 10,000 mol wt). The active principle(s) in NT-F is especially interesting in view of its low molecular weight (around 1000 mol wt) and the possession of antiviral activity in tissue cultures as well as in mice. At present, it is not known whether tobacco smoke condensate itself also contains the antiviral substances.

Summary. A water-soluble and ethanol-insoluble fraction of tobacco leaf of cigarettes has been found to have antiviral activity against EMC virus infection in mice and tissue cultures, and antilethal activity on LCM virus infection in mice. This activity was compared with poly I:C and tilorone as standard agents.

The lower molecular portion of the tobacco extract inhibited the single cycle of EMC virus reproduction in KB cells even when it was added 2 hr after infection. It also inhibited the cytopathic effects of vesicular stomatitis, polio type 2, reo type 2, and vac-

TABLE VI. Anti-LCM Activity of Tobacco Extracts in Mice: Comparison with Standard Immunosuppressive Drugs and Poly I:C.[a]

Agent	Dose (mg)	No. died/total		Treated/ control (%)	p
		Treated	Control		
NT-W-EP	3[b]	20/50	42/50	48	<.001
	1.5	6/10	9/10	67	.14
	0.8	9/10	9/10	100	
Methotrexate	0.001[b]	28/40	36/40	78	<.05
Cyclophosphamide	0.2[b]	23/40	37/40	62	<.005
Azathioprine	0.3[b]	27/40	35/40	77	<.05
Poly I:C	20 μg	9/10	9/10	100	

[a] Agents were injected sc to 10 g Swiss albino mice 2 days after ic inoculation of LD_{70-90} of LCM virus (strain NY-621); injections were continued twice daily for 3 days. Observation was continued for an additional 9 days.

[b] One maximum nontoxic dose (MNTD); mice showed no apparent toxic signs except some slowing of growth (body wt).

cinia viruses, but not adeno type 12, in KB cells. The higher molecular portion showed no antiviral activity in KB cells, although it showed activity in mice against EMC infection.

Although both the lower and higher molecular portions of tobacco extract protected mice from EMC paralysis after a single pretreatment, neither induced serum interferon.

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