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Inactivation of Viruses intentionally added to Urokinase Samples by Heat-Treatment

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An apprehension for virus contaminant cannot be completely eliminated from urokinase (plasminogen activator) preparation originated from human urine theoretically. In this experiment the effect of heat-treatment of urokinase at 60° for 10 hr on infectivity of viruses added intentionally to urokinase samples was investigated.

(1) Ten kinds of viruses used were completely inactivated within 5 hr of incubation at 60°.

(2) Decrease of urokinase activity due to heat-treatment was avoided by adding albumin as a stabilizer.

Keywords——urokinase; heat-treatment; virus inactivation; thermal stability; albumin addition

Urokinase (UK) is an enzyme with fibrinolytic activity (plasminogen activator) isolated from fresh urine of healthy men. UK has been reported to be effective for cerebral thrombosis,[2] myocardial infarction[3] and arteriovenous thrombosis in the limb,[4] and to potentiate the effect of anti-tumor drugs.[5] Though UK is highly purified enzyme, an apprehension about virus contaminant such as hepatitis B virus cannot be completely eliminated because the source of raw material of UK is human urine. Heat-treatment at 60° for 10 hr has been already enforced to some products derived from human blood such as albumin to inactivate hepatitis virus and brought a desired result. Although there have been some reports[6] on the susceptibility of viruses to heat-treatments, the conditions of heating were manifold and it was difficult to conjecture viral sensibility to heat-treatments at 60° from these results.

This paper describes an investigation of the effect of heat-treatment at 60° for 10 hr on activity of viruses added intentionally to UK samples. Hepatitis B virus was not used because of its difficulty in virus assay as well known.

Materials and Methods

Urokinase——Two kinds of UK samples (The Green Cross Corporation, Osaka, Japan) with different activity were used. One sample had a 10000 international unit (IU)/mg of UK activity and the other had a 15000 IU/mg activity, and they were designated as UK-1 and UK-2, respectively. Each UK sample was

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divided into two parts and they were used with or without 0.5% human albumin (The Green Cross Corp.) as a stabilizer for heating. Albumin concentration was decided according to the results of preliminary experiments (unpublished data).

Cells and Viruses——Growth medium for FL, VERO and BHK-21 cells was Eagle's minimal essential medium (MEM) with 10% inactivated fetal calf serum and for HeLa cells was YLE medium (Earle's solution containing 0.1% yeast extract and 0.5% lactalbumin hydrolysate) with 10% fetal calf serum. Cell maintenance medium for virus infection had the same composition except that the serum concentration was reduced to 2%.

Variola major virus (strain Harvey) was grown in HeLa cells and its infectivity was titrated on HeLa cells by focus forming assay.[7] Mumps virus (EC-2a strain), Measles virus (Toyoshima strain) and Japanese encephalitis virus (Sonobe strain, JE) were propagated in VERO cells and Polio virus (type 1), Coxsackie B virus (type 5) and Echo virus (type 6) were grown in HeLa cells, and infectivities of these 6 different viruses were titrated by observations of cytopathic effect (c.p.e.) in respective cell cultures. Vesicular stomatitis virus (Indiana strain, VSV) and Chikungunya virus (BaH 306 strain, CHV) were propagated in FL and VERO cells, respectively, and were titrated in each cells by plaque technique. Rubella virus (M-33 strain) was passaged through BHK-21 cells and assayed for plaque formation in VERO cells. JE, Polio, Coxsackie and Echo viruses were kindly obtained from Dr. M. Tokuda, Institute for Virus Research, Kyoto University.

Heat-Treatment of UK Samples——UK solutions (3000—3500 IU/ml) mixed with one-tenth volume of virus suspensions in 0.05 M phosphate buffer (pH 7.1) were heated. At the same time, viruses suspended in the same buffer were also incubated. After incubation at 60° for 0, 1, 5 and 10 hr, aliquots of these mixtures were removed and the survival virus titers were determined. UK activities at 0 and 10 hr of incubation were also determined by the method of Ploug and Kjeldgaard.[8]

Table I. Effect of Heat-Treatment at 60° on Viral Infectivity (1)

Viruses	Urokinase samples	Viral infectivities/0.2 ml			
		0 hr	1 hr	5 hr	10 hr
Variola virus	UK-1+Alb.[a]	1.4×10^5	0	0	0
	UK-1	(f.f.u.)[c]	0	0	0
	UK-2+Alb.		0	0	0
	UK-2		0	0	0
	Control[b]		0	0	0
Mumps virus	UK-1+Alb.	$10^{6.0}$	0	0	0
	UK-1	($TCID_{50}$)[d]	0	0	0
	UK-2+Alb.		0	0	0
	UK 2		0	0	0
	Control		0	0	0
Measles virus	UK-1+Alb.	$10^{5.5}$	0	0	0
	UK-1	($TCID_{50}$)	0	0	0
	UK-2+Alb.		0	0	0
	UK-2		0	0	0
	Control		0	0	0
Vesicular stomatitis virus	UK-1+Alb.	2.6×10^5	0	0	0
	UK-1	(p.f.u.)[e]	0	0	0
	UK-2+Alb.		0	0	0
	UK-2		0	0	0
	Control		0	0	0
Chikungunya virus	UK-1+Alb.	5.0×10^6	5.0×10^1	0	0
	UK-1	(p.f.u.)	2.5×10^1	0	0
	UK-2+Alb.		2.0×10^1	0	0
	UK-2		4.0×10^1	0	0
	Control		3.5×10^1	0	0

a) Human albumin was added at 0.5%.
b) Viruses were suspended into 0.05 M phosphate buffer, pH 7.1.
c) f.f.u.: focus forming unit.
d) $TCID_{50}$: 50% tissue culture infective dose.
e) p.f.u.: plaque forming unit.

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Results and Discussion

In this experiment four kinds of UK samples (UK-1 +albumin, UK-1 alone, UK-2+albumin, and UK-2 alone) and 0.05 M phosphate buffer (pH 7.1) were used. When ten kinds of viruses in four UK solutions respectively and in phosphate buffer solutions as control were incubated at 60° for 10 hr, all viruses lost their infectivities within 5 hr of incubation (Table I and Table II). Poxvirus group (Variola), paramyxovirus group (Mumps and Measles) and rhabdovirus group (VSV) were easily inactivated within 1 hr incubation. While, togavirus group (CHV, JE and Rubella) and picornavirus group (Polio, Coxsackie and Echo) contained approximately $10^{0.3}$ to $10^{1.5}$ (plaque forming unit, or 50% tissue culture infective dose) per 0.2 ml of infective viruses even after 1 hr incubation at 60°, suggesting that viral susceptibility to heating might differ in different virus groups. Nevertheless, all viruses used were completely inactivated within 5 hr incubation at 60°. The effectiveness under 60° on viral infectivity was not examined.

No difference in survival virus titer after 1 hr incubation was observed among four kinds of UK samples and phosphate buffer solution examined.

There have been reports[9] on the effectiveness of heat-treatment at 60° for 10 hr to inactivate hepatitis virus suspended in human albumin solution. However, all of them were

Table II. Effect of Heat-Treatment at 60° on Viral Infectivity (2)

Viruses	Urokinase samples	Viral infectivities/0.2 ml			
		0 hr	1 hr	5 hr	10 hr
Japanese encephalitis virus	UK-1+Alb.[a]	$10^{5.5}$	$10^{1.5}$	0	0
	UK-1	($TCID_{50}$)[c]	$10^{1.5}$	0	0
	UK-2+Alb.		$10^{1.0}$	0	0
	UK-2		$10^{1.5}$	0	0
	Control[b]		$10^{1.5}$	0	0
Rubella virus	UK-1+Alb.	3.5×10^{5}	0.75×10^{1}	0	0
	UK-1	(p.f.u.)[d]	1.5×10^{1}	0	0
	UK-2+Alb.		0	0	0
	UK-2		1.0×10^{1}	0	0
	Control		0	0	0
Polio virus	UK-1+Alb.	$10^{6.0}$	$10^{1.0}$	0	0
	UK-1	($TCID_{50}$)	$10^{1.0}$	0	0
	UK-2+Alb.		$10^{0.6}$	0	0
	UK-2		$10^{1.0}$	0	0
	Control		$10^{0.6}$	0	0
Coxsackie virus	UK-1+Alb.	$10^{6.5}$	$10^{1.0}$	0	0
	UK-1	($TCID_{50}$)	$10^{0.6}$	0	0
	UK-2+Alb.		$10^{1.0}$	0	0
	UK-2		$10^{0.5}$	0	0
	control		$10^{0.5}$	0	0
Echo virus	UK-1+Alb.	$10^{5.5}$	$10^{0.5}$	0	0
	UK-1	($TCID_{50}$)	$10^{0.3}$	0	0
	UK-2+Alb.		$10^{0.5}$	0	0
	UK-2		$10^{0.6}$	0	0
	Control		$10^{0.5}$	0	0

a), *b*), *c*) and *d*) as in Table I.

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clinical works. The present results have indicated that if viruses strayed into urine, from which UK was derived, viruses were inactivated during the course of incubation at 60° for 10 hr.

Table III shows that when UK-1 and UK-2 samples without the use of albumin were incubated at 60° for 10 hr, their enzymatic activities decreased by approximately 20 to 30% of the original ones, whereas the addition of albumin prevented UK from decreasing its activity during the course of heat-treatment.

Table III. Effect of Heat-Treatment at 60° on Urokinase Activity

Urokinase samples[a]	Residual UK activity[b] at	
	0 hr	10 hr
UK-1+0.5% albumin	100(%)	100(%)
UK-1 alone	100	83
UK-2+0.5% albumin	100	94
UK-2 alone	100	69

a) UK-1 and UK-2 showed 3000 IU/ml and 3500 IU/ml activity, respectively.
b) Average of three experiments.

The present studies show that UK samples prepared as described above possessed high enzymatic activity and were free from infective viruses.

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