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DISRUPTION OF INFLUENZA VIRUS A BY DIETHYLETHER-TWEEN AND TRI-N-BUTYL PHOSPHATE-TWEEN MIXTURES

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Summary. — In search for optimal conditions of influenza virus A/Brazil/78(H1N1) disruption by diethylether-Tween 80 and tri-n-butyl phospate (TNBP)-Tween 80 mixtures, the following treatments were found suitable: for 120 min at 4 °C with 3.3% TNBP and 0.1% Tween 80 or for 120 min at 4 °C with diethylether and 0.1 % Tween 80 (ratio of diethylether and treated virus material 1 : 1). Disruption by TNBP appeared more favourable not only because of the convenient performance but also due to the higher antibody-inducing ability of the product obtained. The suggested removal of TNBP from the disruption product by extraction into hexan is easy and reliable. Chemical analysis enabled precise detection of 0.1% TNBP in the "vaccine" product. The hexan-extracted "vaccine" contained less than 0.05% TNBP, a concentration non-toxic for mice.

Key words: influenza A virus; disruption; diethylether; tri-n-butyl phosphate; vaccine

Introduction

Recent efficient methods of virus concentration and purification and the development of subunit vaccines obtained by the virus disruption allowed to decrease the side effects of influenza vaccines. Commercial production of subunit vaccines employes lipid solvents for disruption of influenza virus, such as Triton-X100 (Connaught Lab.), cetyltrimethyl-ammonium bromide (Sandoz Pharmaceuticals), TNBP-Tween (Weyth Lab.) or diethylether-Tween (Parker-Davis and Co.) mixtures or diethylether only (Immuna Czechoslovakia). Mere diethylether disruption, however, results in a marked decrease in active haemagglutinin (HA) content, especially with H1 strains. Due to the contemporary need of influenza vaccines possessing H1 and H3 HA, the use of diethylether has become unsatisfactory for the vaccine preparation. Therefore, we tried to find optimal conditions of influenza virus A disruption preserving intact both HA and neuraminidase (NA).

Materials and Methods

Influenza virus A/Brazil/78(H1N1) was grown in 10-day-old chick embryos. Virus containing allantoic fluids were concentrated by high speed centrifugation (90 min/150,000 g) and purified by sucrose density gradient (20—60%) centrifugation. The fraction with the highest HA activity was separated from sucrose and further concentrated by high speed centrifugation (90 min/ /150,000 g).

Haemagglutination and haemagglutination-inhibition tests were carried out on plexiglass panels according to Raška (1958). Nonspecific inhibitors were removed from sera by receptor destroying enzyme (RDE) as described (Davenport and Minuse, 1964).

Neuraminidase test was performed by the slightly modified method of Russ *et al.* (1974), in which incubation in the absence Ca^{2+} was prolonged to 18 hr.

Immunogenicity of disruption products was tested on outbred white mice weighing 12 and 18 g from the breed in the first and in the second experiment, respectively. Sera from individual groups of intraperitoneally (i.p.) inoculated mice were pooled, stored at -20 °C and examined by HI test simultaneously.

Protein concentrations were determined by the method of Lowry *et al.* (1951).

Removal of ether was described before (Závadová *et al.*, 1975).

Estimation of TNBP in water solution was performed according to Pan and Grebennikov (1976).

Thin-layer chromatography was carried out on Silufol plates (starch-connected silicapearls on aluminium sheet; Kavalier Votice, Czechoslovakia). TNBP mobility was determined by the acid detection method of phosphate groups according to Bandurski and Axelrod (1951).

Results

In preliminary experiments, diethylether only was used for disruption of influenza virus A/Brazil/78(H1N1) according to the procedure elaborated for preparation of H3N2 influenza virus subunit vaccine (Závadová *et al.*, 1975).

Table 1. Conditions of influenza A virus disruption and HA titres in original and disrupted samples

Lipid solvent	Detergent	Time of disruption (min)	Cooling	HA titre in samples	
				original	disrupted
ether 1 : 1		60	—	2,560	20
	Tween 80	60	—	2,560	320
	0.1%	60	+	2,560	320
		120	—	2,560	2,560
		120	+	2,560	2,560
	0.1%	120	+	5,120	5,120
TNBP 0.1%	Tween 80	60	—	5,120	5,120
	1.0%	120	+	5,120	5,120
3.3%		60	—	2,560	320
		60	+	5,120	10,240
		120	—	2,560	1,280
		120	+	5,120	5,120
	0.02%	120	—	16,000	8,000
		120	—	5,120	5,120
30%	0.1%	60	—	3,200	2,560
		120	—	2,560	320
		120	+	5,120	2,560

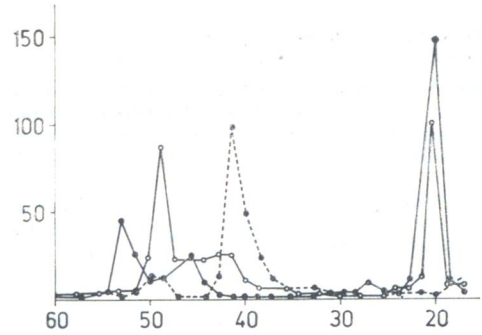

Fig. 1.
The rate of disruption of virions as determined by HA titre in fractions from sucrose gradient (centrifugation for 90 min at 150,000 g)
(●) Disruption for 120 min under cooling with 3.3% TNBP and 0.1% Tween 80 (○) or with diethylether and 0.1% Tween 80(ratio of diethylether and treated virus material 1 : 1); ● --- ● intact virus. Abscissa: % of sucrose concentration. Ordinate: HA titre related to HA titre of original virus sample which was considered as 100%

Because such treatment resulted in a marked decrease in HA activity of the H1N1 strain tested, in further experiments either Tween 80 was added to diethylether or the influenza virus was treated with the TNBP-Tween 80 mixture. Disruption was carried out in an icy bath under stirring. HA titres in the original and disrupted samples and the conditions of influenza virus A disruption are given in Table 1.

The rate of virion disruption to subunits was determined by HA test in the fractions harvested from sucrose gradient centrifugation. Based on the results obtained, optimal disruption was achieved by treatment for 120 min under cooling with diethylether and 0.1% Tween 80 (ratio of diethylether and treated virus material 1 : 1) or with a combination (v/v) of 3.3% TNBP and 0.1% Tween 80 (Fig. 1). In both, the TNBP and diethylether-disrupted virus samples, two peaks of HA activity were observed respectively — one corresponding to 20%, another to 50% sucrose concentration; the maximum HA activity of untreated virus was in the 40% sucrose concentration.

Investigation of the effect of influenza virus A disruption on neuraminidase activity of the products obtained revealed no substantial difference in individual samples, it was somewhat higher after TNBP-Tween 80 than after diethylether-Tween 80 treatment (Table 2).

The immunogenicity for mice of intact virus and disruption products is compared in Table 3. In the first experiment mice were inoculated i.p. with 0.2 ml vol containing 400 or 4,000 HA units/ml and serum was harvested on days 3, 6, 10, 14, 21 and 28; in the second experiment they received

Table 2. The results of neuraminidase testing in intact and disrupted samples

Virus sample	Neuraminidase titre in batch No.			
	1	2	3	4
original	1 : 66	1 : 76		1 : 92
disrupted by TNBP (3 : 3%)	1 : 132	1 : 135	1 : 28	1 : 134
by diethylether	1 : 97	1 : 123	1 : 29	1 : 115

Table 3. Titres of HI antibodies to influenza virus A/Brazil/1978 strain in sera of mice immunized with intact and TNBP or diethylether-disrupted virus

HA unit/ml	Virus sample	HI serum antibody titres on days post immunization						
		3	6	10	14	21	28	35
400	intact	0	0	10	10	40	40	
	TNBP-treated	0	10	20	40	40	40	
	diethyl-ether-treated	0	0	20	30	40	40	
4 000	intact	10	10	10	100	100	100	
	TNBP-treated	0	30	80	80	100	100	
	diethyl-ether-treated	0	0	15	20	20	80	
10 000	intact				80	80	80	160
	TNBP-treated				80	160	160	160
	diethyl-ether-treated				20	20	30	60
20 000	intact				40	160	160	160
	TNBP-treated				160	160	160	160
	diethyl-ether-treated				20	20	40	80

0.5 ml volumes containing 10,000 or 20,000 HA units/ml and serum was harvested on days 14, 21, 28 and 35, respectively. The level of serum HI antibody titres to influenza virus A/Brazil/78 was found lower following the inoculation of diethylether-Tween 80-treated than of TNBP-Tween 80--treated virus. The TNBP-Tween 80-disrupted virus product elicited similar HI antibody response as did the intact virus.

Data on neurotoxicity of TNBP (Johannsen *et al.*, 1977) led us to test the toxicity of this chemical. Three mice in each group were inoculated i.p. with 0.5%, 0.1%, 0.05%, 0,01% and 0.005% v/v concentrations of TNBP in phosphate buffer containing 0.1% Tween 80 in 0.5 ml volumes. Higher concentrations, namely 0.5%, 0.1% and 0.05% TNBP killed mice immediately after inoculation. In contrast, all mice inoculated with 0.01% and 0.005% TNBP survived for 6 weeks period of observation.

Because of the toxicity of TNBP, we tried to remove it from the final "vaccine" product. Besides other methods, the possibility of TNBP ex-

Table 4. Detection of different concentration of TNBP before and after hexan extraction of influenza virus TNBP-Tween 80 mixture

TNBP concentration	OD_{700} before hexan extraction		OD_{700} after hexan extraction	
0.001%	0.007	0.009	0.026	0.032
0.005%	0.010	0.008	0.141	0.127
0.01%	0.008	0.012	0.258	0.248
0.02%	0.009	0.008	unmeasurable	
0.05%	0.007	0.008	unmeasurable	
0.1%	0.014	0.010	unmeasurable	

Table 5. Detection of TNBP in the "vaccine" product following separation of TNBP by thin-layer chromatography

Sample	OD_{700}		
Concentration of TNBP in butanol			
blank (control)	0.065	0.064	0.066
1%	0.551		
0.1%	0.206	0.212	0.215
0.05%		0.119	0.122
0.01%	0.072		
0.001%	0.069		
Concentration of TNBP in nonpurified vaccine			
3.3%	0.604	0.415	
1%	0.204	0.207	
0.1%	0.183	0.180	0.187
0.05%		0.072	0.064
Vaccine after hexan extraction	0.064	0.069	0.066
Diethylether-treated virus	0.070	0.068	0.068

traction followed from its properties. Hexan appeared as a suitable extracting substance, since it is low reactive, non-mixing with water and water insoluble, of low viscosity and density differing from that of the water, i.e. it is easily separable from the water phase. It did not decrease HA and neuraminidase activities in the samples tested and, moreover,it is considered for a non-toxic substance (Foa *et al.*, 1976).

The proper extraction was carried out by shaking TNBP-treated virus preparation with hexan. Decanted material was inoculated i.p. into mice which all survived. To find out whether TNBP can be removed from PFR by hexan, method of TNBP detection according to Pan and Grebennikov (1976) was used. No TNBP either in the solution or in the mixture with PFR (in the presence of 0.1% Tween 80, v/v) could be detected after hexan extraction (Table 4).

Detection of TNBP directly in the disrupted virus is impossible, because it requires reaction with phosphate groups, which are present also in the virus material. For separation of TNBP from the "vaccine", thin layer chromatography on the starch-connected silicapearls was employed. Butanol was added to the virus-PFR-TNBP-Tween sample following disruption and 75 μl volume of resulting mixture was layered on the plate. After development in butanol and ethyl acetate mixture and drying of the plate, the material was scratched from the place to which TNBP migrated. Water was poured on the sample and after several hours TNBP was determined in the water phase as described (Pan and Grebennikov, 1976). As follows from Table 5, the hexan-extraction procedure used removed TNBP from the virus material below 0.1% concentration.

Discussion

In search for optimal conditions of influenza virus A/Brazil/78(H1N1) disruption to prepare subunit vaccine, we started with commercially used disruptive chemicals, which could supposed to be harmless from the public health standpoint. As suitable were proved diethylether-Tween 80 and TNBP-Tween 80 mixtures, providing that diethylether and TNBP, respectively, were removed. We did not concern with a removal of Tween 80, because this substance is widely used in food and cosmetic industry (Marshall, 1973). For influenza virus disruption the optimal treatments found were either 3.3% TNBP and 0.1% Tween 80 for 120 min under cooling or diethylether and 0.1% Tween 80 mixtures (ratio of diethylether and treated virus material 1 : 1).

We repeatedly observed an increase in HA titre after virus disruption. We suppose that this might be caused by removal (decrease) of steric limitation during antigen-erythrocyte interaction. Of interest was also the finding of two peaks of HA activity in analysed samples of disrupted virus in sucrose gradient. The appearance in higher density of the peak of HA activity from the TNBP-Tween-disrupted virus than from the intact virus was found also by Neurath (1970), who suggested that position of subunits of disrupted virus (A_2/Japan/62a/Taiwan/66) in higher specific density of CsCl equilibrium gradient was due to removal and separation of lipids, respectively, from the HA during virus disruption.

Uncertain was the removal of TNBP from the "vaccine" product. Technique recommended in the literature, such as decantation (Neurath, 1976), centrifugation for 10 min at 14,000 g followed by gel filtration (Collins, 1978) were found either tedious or not too reliable. For this reason we used the own procedure of TNBP removal by extraction into hexan. The degree of TNBP removal was checked by two tests — chemical evaluation of the quality of extraction and toxicity testing on white mice. The former test consisting in thin-layer chromatography and detection of phosphate groups (Pan and Grebennikov, 1976), made it possible to determine 0.1% TNBP concentration in disrupted virus material. By the latter test we were able to detect 0.05% TNBP concentration, which was still lethal for white mice. The final "vaccine" product, from which TNBP was extracted into hexan, contained surely less than 0.05% TNBP.

Finally, for preparation of subunit vaccine from influenza virus strains of H1HA type we recommended the treatment with TNBP-Tween 80 mixture, because it provides higher immunogenicity of the "vaccine" product, and its use and removal during the commercial preparation of the "vaccine" is much more safe than that of diethylether-Tween 80 mixture. Nitrogen bubling used for the removal of diethylether remains questionable, when TNBP for virus disruption should be employed.

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