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Effects of Tween 80 and Freon 113 on Measles Virus

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Measles vaccines were prepared from the same virus fluids by inactivation with formaldehyde or by extraction with ether, ethyl acetate, or Freon 113 in the presence of Tween 80. Tests of antigenic potency, based on antibody levels in guinea pigs, showed that the formaldehyde-inactivated vaccines were more potent than the solvent-inactivated preparations and had the additional advantage of long shelf life. Residual Tween 80 in the solvent-extracted vaccines resulted in marked loss of immunogenic potency without significant loss of hemagglutinating activity. Neither extraction with organic solvents nor exhaustive dialysis efficiently removed Tween 80 from aqueous solutions.

Two methods for the preparation of inactivated measles vaccine are currently in use. In Canada and the United States, inactivation of the virus by formaldehyde has been the method of choice, but in Europe inactivation by organic solvents has been used. Norrby et al. (7) prepared vaccine by inactivating the virus by treatment with polysorbate 80 (Tween 80) and diethyl ether (TE vaccine) and, based on extinction limit titers in guinea pigs, found their TE vaccine to be three to four times more potent than an alum-precipitated formaldehyde-inactivated vaccine prepared in the United States. This comparison was made with vaccines prepared from different virus fluids, processed by different manufacturers, and does not, therefore, provide information on the relative merits of the two methods of inactivation.

As ethyl ether is a poor lipid solvent and relatively ineffective in denaturing nonviral proteins, its use in purifying viral vaccines is limited. Its efficiency in purifying viral hemagglutinin is increased, however, by the addition of a detergent such as Tween 80 (5). Gessler et al. (1) showed that greater purification of viruses was achieved by extraction with halogenated fluorocarbons, particularly trichlorotrifluoroethane (Freon 113); because this solvent has the additional advantage of being less hazardous in large-scale operations, its effects on measles virus have been studied. A small number of vaccines prepared by extraction with ether or ethyl acetate have been included for comparison.

The experiments described in this report were designed to determine whether Tween-solvent-inactivated measles vaccines would have advantages over formaldehyde-inactivated vaccines and were extended to include observations on the effects of Tween 80 alone. Appraisal of the various preparations has been based on antigenic potency and stability.

Further attempts have been made to obtain correlation between in vitro measurements of antigenic potency, such as hemagglutination (HA) and complement fixation (CF), and immunogenic response in animals. In the latter method, relative potencies have been determined from estimations of ED_{50} and geometric mean antiserum titers. Both tests are subject to wide deviations and this, of course, makes correlation difficult.

MATERIALS AND METHODS

Measles virus fluids. The Edmonston strain of measles virus was grown in primary cultures of monkey kidneys. When the cell sheet was almost completely destroyed by cytopathogenesis and the HA titer was at a maximum, the cultures were frozen and thawed once and pools of approximately 10 liters each were prepared from a number of culture vessels. The pools were subjected to treatment with ultrasonic vibration for 1 hr. The latter procedure approximately doubled the HA and CF titers.

Concentration of live or inactivated virus. The virus was concentrated 10- to 20-fold by adsorption to calcium phosphate, and solution of the adsorbant was effected in a small volume of ethylenediaminetetraacetic acid (EDTA) by the method described by Macmorine and Parisius (3). Concentrated virus was dialyzed twice against 10 volumes of 0.85% sodium chloride and once against 10 volumes of medium 597 Rec (3) to remove excess EDTA and calcium complexes.

Inactivation of virus with formaldehyde. Cellular debris was removed from the virus fluids by passage through filters of high porosity before any inactiva-

tion procedure was carried out. The virus was inactivated at 37 C for 46 hr in the presence of formaldehyde (1 part USP solution per 4,000 parts of vaccine).

Inactivation with Tween 80 and solvents. A 5% aqueous stock solution of Tween 80 (Atlas Chemical Industries, Brantford, Ont., Canada) was prepared and sterilized by autoclaving for 30 min at 128 C. After adding various amounts to the virus fluids, the mixtures were incubated for 30 min at 37 C and for a further 1.5 hr at room temperature, unless otherwise indicated. Maximum activity of Freon 113 was found with a volume of 20% (v/v), and this ratio of solvent to virus fluid was used in all experiments. To inactivate the virus, Freon 113 and virus fluids were mixed vigorously for 24 hr at room temperature. The phases were separated by centrifugation and the aqueous phase dialyzed.

Assay of Tween 80. MacAllister and Lisk's method (2) for the assay of polyoxyethylene stearate was adapted to the assay of Tween 80 and was as follows. Acetic acid (800 ml, 0.025 N) was adjusted to *p*H 5.0 with sodium hydroxide and diluted to 1,000 ml with distilled water; 0.40 g of purified potato starch was then suspended in 500 ml of the acetate solution. After boiling gently for 5 min, the fluid was cooled and filtered through one layer of paper (Reeve Angel 202); 1 ml of 37% formaldehyde was added as a stabilizer, and the solution was diluted to 1,000 ml with distilled water.

To prepare the iodine stock solution, iodine (2.50 g) and potassium iodide (5.00 g) were dissolved in distilled water and diluted to 500 ml. For use in the test, a fresh dilution of 1 part iodine stock solution in 200 parts of distilled water was prepared daily.

To prepare a Tween 80 standard stock solution, 10.00 g of Tween 80 was dissolved in warm distilled water and diluted to 1,000 ml. This solution was diluted 1 to 10 to give a concentration of 1 mg of Tween 80 per ml.

Standard curve. A set of standard solutions containing 0.020, 0.015, 0.010, and 0.005 mg (per ml) of Tween 80 and a blank were prepared. To 1.0 ml of the solutions, in test tubes (13 by 100 mm), 1.0 ml of starch solution and 1.0 ml of diluted iodine solution were added. Five minutes after mixing, the percentage transmission was measured in a Coleman Junior spectrophotometer at a wave length of 700 μm, having set the blank to read 40%, and a standard curve was prepared. Samples of unknowns were diluted to contain between 0.005 and 0.015 mg of Tween 80 per ml and treated in the same way.

Tests for antigens. The CF procedure was essentially the same as that described by Macmorine et al. (4) for measurement of poliovirus antigens and has already been described (3).

Basically, the HA test was performed by the method of Rosen (8). Twofold serial dilutions of the sample were prepared; to each 0.2 ml of diluted sample, 0.2 ml of a 0.5% suspension of *Cercopithecus* monkey erythrocytes and 0.2 ml of phosphate-buffered saline were added. The tests were read after 2 hr at 37 C. The highest dilution with definite HA was taken as the end point.

To determine antigenicity in guinea pigs, samples were tested by the use of three dilutions of vaccine, including undiluted, and 10 or more animals per dilution. Each guinea pig received a single dose of 0.5 ml intramuscularly and was bled 3 weeks later. After adsorption with monkey erythrocytes, each serum was tested for HA-inhibiting antibodies. This was done by a microtest in which 0.025 ml of diluted serum was mixed with 0.025 ml of challenge antigen, containing 4 HA units of measles antigen, and 0.025 ml of a 0.5% suspension of *Cercopithecus* erythrocytes. Except for these details, Rosen's method was used.

RESULTS

An initial experiment on inactivation by three solvents in the presence of Tween 80 (1.25 mg/ml) was carried out on a single virus pool, concentrated 10-fold by the calcium phosphate-EDTA procedure. Mixing of the phases was continued for 15 min at 4 C with ether and ethyl acetate and for 24 hr at room temperature with Freon 113. Extracted concentrates were reconstituted to give 2.5-fold vaccines based on volume. A control was prepared by treating unconcentrated virus with formaldehyde, followed by concentration, dialysis, and reconstitution. None of the three solvent-extracted preparations was significantly different from the formaldehyde-inactivated vaccine nor from each other (Table 1).

A further comparison of formaldehyde and Tween 80-Freon 113 inactivation was made on four different measles virus pools (Table 2). Although it was considered advantageous to extract small volumes of concentrated virus, to obviate the handling of large volumes of solvent, one vaccine was prepared by extracting unconcentrated virus fluid. It was observed that contact between virus and solvent for 24 hr at room temperature did not in all cases completely destroy virus infectivity, but continuation of contact beyond this period reduced the CF and HA titers. An attempt to achieve complete inactivation of live virus was made by pretreatment of the virus fluid with Tween 80 (0.3 mg/ml) at 4 C. After 12 days, the fluids were concentrated and extracted. This procedure gave complete inactivation of the infectivity of the virus. A formaldehyde-inactivated vaccine was prepared from each virus pool, and in Table 2 the results obtained with the solvent-treated vaccines are expressed as antigenic potencies relative to the corresponding formaldehyde-inactivated vaccine. A statistical analysis indicated that the CF titer was not raised significantly by the solvent treatments. The mean relative HA potency of 3.9 is significantly greater than 1 and is in good agreement with results reported for treatment with Tween 80 and ether. With samples A to E, there was no significant difference between the relative

potencies based on geometric mean titers. They were, therefore, treated as a single group, and it was found that the formaldehyde-inactivated vaccines were, on the average, more potent than the solvent-extracted preparations. Based on immunogenic response in guinea pigs, vaccines F, G, and H were of lower potency than vaccines A to E, in spite of their high HA titers. The observations on these three preparations raised the question of the stability of measles vaccine containing as little as 0.3 mg of Tween 80 per ml, as it was assumed that a high concentration of this detergent would remain in the aqueous phase after solvent extraction.

TABLE 1. *Comparison of three solvent-inactivated virus fluids and a formaldehyde-inactivated control*

Preparation of vaccine	CF (units/ml)	HA (units/0.2 ml)	Potency relative to reference vaccine[a]	
			ED_{50}[b]	GM[c] titer (95% limits)
Tween 80-ether..	9	128	4.3	7.2 (5.0–10.7)
Tween 80-Freon 113.....	13	256	2.0	4.0 (2.8–5.8)
Tween 80-ethyl acetate.....	10	128	3.0	4.8 (3.4–7.0)
Formaldehyde..	10	56	4.3	6.0 (4.2–8.8)

[a] Connaught Medical Research Laboratories lot 101.
[b] Extinction limit titer.
[c] Geometric mean.

Three vaccines described in Table 2 were stored as 10-fold concentrates at 4 C and tested after 3.5 and 5 months. Although the CF and HA antigens were relatively stable, the vaccines prepared by solvent extraction were unstable (Table 3). The results of the animal tests were equivocal for the formaldehyde-inactivated control, as an unusually high potency was found on its initial test. However, after both 3.5 and 5 months of storage, the formaldehyde-inactivated control was of higher potency than the solvent-extracted vaccines.

Distribution studies on Tween 80 in Freon, ether, and ethyl acetate aqueous systems were undertaken. Aqueous solutions containing Tween 80 (1 mg/ml) were extracted three times with Freon 113 (20% v/v), ether, or ethyl acetate by vigorous shaking for 10 min. After each extraction, the phases were separated by centrifugation for 10 min at 1,200 rev/min, and the aqueous phases were assayed for Tween 80. Even after three extractions with Freon, 80% of the Tween remained in the aqueous phase, with ether 90%, and with ethyl acetate 55% (Table 4).

The efficiency of dialysis for removal of Tween 80 from an aqueous solution was also determined. For this, three successive 10-fold concen-

TABLE 2. *Comparison of solvent- and formaldehyde-inactivated vaccines*

Vaccine	Description of sample and sequence of treatment[a]	Concn of Tween 80	Potency relative to the corresponding formaldehyde-inactivated control			
			CF	HA	Antigenicity tests in guinea pigs	
					ED_{50}[b]	GM[c] (95% limits)
		mg/ml				
A	From concentrated live virus	1.25	1.3	4.0	0.5	0.6 (0.4–0.9)
B	From concentrated live virus (repeat of above)	1.25	1.1	4.0	0.7	0.5 (0.3–0.7)
C	From concentrated live virus	0.63	1.3	2.6	0.5	0.9 (0.5–1.7)
D	From concentrated live virus	0.31	1.9	2.0	0.9	0.8 (0.2–2.1)
E	Unconcentrated live virus, concentrated after treatment	1.25	2.7	1.6	0.7	0.9 (0.5–1.6)
F	Unconcentrated live virus + Tween 80 (0.31 mg/ml), stored for 12 days at 4 C, concentrated, and treated	1.25	1.4	4.0	0.3	0.2 (0.1–0.3)
G	Pretreated fluid as in F	0.63	4.8	4.0	0.6	0.2 (0.04–0.6)
H	Pretreated fluid as in F	0.31	2.3	7.6	0.2	0.1 (0.02–0.4)

[a] All solvent-inactivated fluids were extracted with 20% (v/v) Freon 113 and reconstituted to give a concentration equivalent to 2.5-fold based on original volume. The formaldehyde-inactivated vaccines were concentrated and reconstituted in the same way.
[b] Extinction limit titer.
[c] Geometric mean titer.

TABLE 3. *Stability of formaldehyde- and Tween-Freon-inactivated measles vaccines stored as 10-fold concentrates at 4 C*

Description of samples[a]	In vitro tests		Antigenicity tests in guinea pigs, potency relative to reference vaccine	
	CF (units/ml)	HA (units/0.2 ml)	ED_{50}[b]	GM[c] (95% limits)
Formaldehyde-inactivated control	28	128	8.9	17.8 (10.2–33.1)
After 3.5 months of storage at 4 C		128	5.8	3.7 (2.2–6.5)
After 5 months of storage at 4 C	28	256	4.5	6.1 (4.2–9.5)
Vaccine B (*see* Table 2)	32	512	5.9	7.9 (4.7–13.9)
After 3.5 months of storage at 4 C		512	1.6	1.3 (0.7–2.2)
After 5 months of storage at 4 C	34	512	2.3	2.9 (2.0–4.3)
Vaccine F (*see* Table 2)	38	512	2.8	3.2 (1.9–5.4)
After 3.5 months of storage at 4 C		512	1.2	0.5 (0.3–0.9)
After 5 months of storage at 4 C	34	256	1.2	1.4 (1.0–1.9)

[a] All vaccines were reconstituted to 2.5-fold (based on volumes) for testing.
[b] Extinction limit titer.
[c] Geometric mean titer.

TABLE 4. *Extraction of Tween 80 from aqueous solutions by three organic solvents (20%, v/v)*

Solvent	No. of extractions	Tween 80 in aqueous phase after extraction[a]
		mg/ml
Freon 113	1	1.00
	2	0.85
	3	0.80
Ether	1	0.95
	2	0.90
	3	0.90
Ethyl acetate	1	0.80
	2	0.70
	3	0.55

[a] Before extraction, each solution contained 1.00 mg/ml.

TABLE 5. *Removal of Tween 80 from aqueous solutions by dialysis against 0.85% NaCl solution (three changes of 10 volumes)*

Concn of Tween 80 (mg/ml)	
Before dialysis	After dialysis
0.01	0.006
0.10	0.067
1.00	0.720

TABLE 6. *Tween 80 levels of the Tween-Freon-treated samples described in Table 2*

Sample	Theoretical concn of Tween (mg/ml)				Concn of Tween by assay
	In 10× concentrated vaccine	After extraction (90% estimated)	After dialysis (65% estimated)	After 1:4 dilution of concentrate	
					mg/ml
A	1.25	1.12	0.73	0.18	0.25
B	1.25	1.12	0.73	0.18	0.18
C	1.25	1.12	0.73	0.18	0.20
D	0.63	0.56	0.36	0.09	0.06
E	0.31	0.28	0.18	0.045	0.03

trations of Tween 80 were dialyzed in 2.54-cm cellulose tubing for 24-hr periods against three changes of 10 volumes of 0.85% sodium chloride. Regardless of the original concentration of Tween 80, 60 to 70% remained in the dialyzing bag (Table 5).

By using the above experimental factors, a comparison between the calculated concentrations of Tween 80 and the determined values was made on a number of the vaccines described in Table 2. The estimated concentrations agreed well with the amounts found on assay (Table 6).

From the above it appeared that Tween 80 by itself could adversely affect the stability of measles antigenicity; to examine this possibility, Tween 80 was added to a virus fluid. In both the control and the experimental fluid, the CF and HA antigens were relatively stable (Table 7). The control showed no loss of immunogenic potency in 6.5 months at 4 C. On the contrary, although there is some evidence that the addition of Tween 80 may have raised the initial potency of the virus fluid, there is no doubt that on continued storage the presence of Tween 80 (0.3 mg/ml) significantly reduced the antigenic response in guinea pigs.

TABLE 7. *Effect of Tween 80 on crude measles virus fluid stored at 4 C*

Description of sample	Storage period at 4 C	In vitro tests		Antigenic potency in guinea pigs, relative to reference vaccine	
		CF (units/ml)	HA (units/0.2 ml)	ED_{50}[a]	GM[b] (95% limits)
Control virus (0.005 mg of Tween per ml)	11 days[c]	15	64	1.8	1.9 (1.4–2.6)
	2.5 months	9	32	2.8	4.7 (2.6–9.5)
	4.5 months	16	64	3.0	2.8 (1.9–4.1)
	6.5 months	17	32	5.9	7.9 (4.2–17.7)
Virus with 0.3 mg of Tween 80 per ml[d]	11 days[c]	22	128	4.4	3.1 (2.2–4.3)
	2.5 months	15	64	0.5	0.8 (0.5–1.4)
	4.5 months	27	64	1.0	1.0 (0.7–1.4)
	6.5 months	25	32	0.9	1.0 (0.6–1.8)

[a] Extinction limit titer.
[b] Geometric mean titer.
[c] Eleven days elapsed before testing in animals could be initiated. These results are considered to be zero-time assays.
[d] The Tween 80 was added to the virus fluid at room temperature. The samples were chilled immediately to 4 C.

DISCUSSION

Measles virus was inactivated by extraction with ether, ethyl acetate, or Freon 113, in the presence of Tween 80, and gave vaccines of good immunogenic potency. Although Freon reduced infectivity more slowly than did ether, possibly as a result of its low solubility in aqueous solutions, no real difference in the resulting vaccines was demonstrated. The main purpose of the study, however, was to determine whether the use of an organic solvent resulted in less damage to the immunogen during inactivation than did formaldehyde. From a number of comparisons on vaccines made from the same virus fluids, it was found that the formaldehyde-inactivated preparations were, on the average, more potent than the solvent-inactivated preparations. In addition, remarkable stability of the formaldehyde-inactivated vaccines was observed. There appeared little doubt that the presence of even 0.3 mg of Tween 80 per ml resulted in marked loss of immunogenicity on storage at 4 C. With Norrby's process, the Tween 80 was removed, in all probability, by passage of the extracted virus through Sephadex G-200 followed by ultracentrifugation, but no assays of Tween nor data on stability of the vaccine were presented by him. Of particular interest was the significant lack of correlation between HA activity and the immune response in guinea pigs, since Norrby suggested that the HA antigen is the one responsible for the stimulation of neutralizing antibodies (6). Our results, based on animal experiments, showed that Tween 80 and Freon 113 could reduce the antigenic potency of experimental vaccines to one-fourth the original value, without decreasing their hemagglutinating activity.

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