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Effect of Formalin, β-Propiolactone, Merthiolate, and Ultraviolet Light Upon Influenza Virus Infectivity, Chicken Cell Agglutination, Hemagglutination, and Antigenicity

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Four strains of influenza virus were treated with Formalin, Merthiolate, Merthiolate and Formalin, ultraviolet light, and β-propiolactone (BPL) for 18, 48, and 72 hr. Infectivity, chicken cell agglutination (CCA), hemagglutination (HA), and antigenicity determinations were made. Except for Merthiolate, each method of inactivation was equally effective in reducing infectivity. Loss of infectivity was related to length of treatment. CCA determinations were higher for all treated groups except for BPL-treated samples; these had lower determinations. BPL treatment also lowered the HA titer. Antigenicity was lessened by BPL treatment and by Merthiolate and Formalin treatment. Generally, the length of inactivation up to 72 hr did not affect CCA, HA, or antigenicity determinations. For the most part, there was no significant differences in the reactivity of the four strains.

There have been numerous studies on the inactivation of viruses for potential vaccine use (2, 3, 6, 7, 8, 10, 11, 12). The methods of inactivation include the use of chemicals (e.g., formaldehyde, β-propiolactone, and ethylene oxide), physical forces [e.g., ultraviolet light (UV)], and a combination of these.

This study was designed to investigate how Formalin, Merthiolate, a mixture of Merthiolate and Formalin, UV, and β-propiolactone (BPL) affect the infectivity, hemagglutination (HA), and antigenicity of representative A, A2, and B strains of influenza virus.

MATERIALS AND METHODS

Viruses. The following influenza strains used in this study were part of the reagent collection maintained in our laboratory: A/PR/8/34, A2/Japan/170/62, A2/Georgia/1/67, and B/Massachusetts/3/66.

Method of preparing virus concentrates. Ten- to 11-day-old chicken embryos were inoculated into the allantoic sac with 0.2 ml of an appropriate dilution of seed virus. After incubation at 36 C, infectious allantoic fluids were harvested 48 hr later for the A and A2 strains and at 64 hr for the B strain. The pooled fluids were first centrifuged at 1,000 × *g* at 4 C for 30 min to remove the heavier debris. The clarified fluids were then centrifuged in a model L-2 Spinco by using an SW25.2 rotor at 53,000 × *g* for 1.5 hr at 4 C. The virus pellet was resuspended in a quantity of 0.01 M phosphate-buffered saline (PBS), *p*H 7.2, equal to 1/10 the original volume of allantoic fluid. To resuspend as much of the pellet as possible, it was necessary to sonic treat the final virus concentrate in a Raytheon sonic oscillator at 10 kc for 30 sec.

Formalin treatment. A 1-ml amount of stock 2% Formalin in PBS was added to 100 ml of virus concentrate. Final Formalin concentration was 1:5,000. Samples of this formalinized concentrate were removed after 18, 48, and 72 hr of incubation at 34 C.

Merthiolate treatment. A 1-ml amount of stock 1% Merthiolate in PBS was added to 100 ml of virus concentrate making a 1:10,000 final concentration of Merthiolate. Samples of this merthiolated concentrate were removed after 18, 48, and 72 hr of incubation at 34 C.

Merthiolate and Formalin. To a 100-ml sample of concentrate was added 1 ml of a stock 2% Formalin-1% Merthiolate solution. After incubation at 34 C, samples were removed at 18, 48, and 72 hr.

Control fluids. A portion of each viral concentrate before treatment was the zero-hour control. A 1-ml amount of PBS was added per 100-ml portion of concentrate. This mixture was incubated at 34 C along with the treated concentrates, and samples were removed at 18, 48, and 72 hr. These served as controls for the Formalin-, Merthiolate-, and Merthiolate-Formalin-treated samples.

BPL treatment. "Betaprone" (Testagar and Co., Inc., Detroit, Mich.) treatment followed the method

of Sever et al. (14). The control for this procedure was 0.85% saline-1.68% sodium bicarbonate-0.2% phenol red solution.

UV light treatment. The virus concentrate was placed in 60-mm petri dishes and positioned to ensure an energy of 4,000 ergs per cm^2 per sec. The fluid which had a depth of 3 mm was exposed for 3 or 6 min. The control was an untreated sample.

Dialysis. The controls and treated samples of the Formalin, Merthiolate, and Merthiolate-Formalin groups were subjected to dialysis immediately after the conclusion of the treatment period. The dialysis was performed with PBS for 3 hr at 4 C.

Infectivity measurement. Ten- to 11-day-old chicken embryos were used to measure infectivity. HA tests were performed on the allantoic fluids after incubation at 36 C for 48 hr for A and A2 strains and for 64 hr for B strains. All infectivity end points were determined by the Kärber method (5).

Chicken cell agglutination (CCA). The method followed was that of Miller and Stanley (9).

HA and hemagglutination-inhibition (HI). The method of Clarke and Casals (1) was modified for use in microtiter equipment (13).

Antigenicity. Guinea pigs (National Institutes of Health-Hartley strain) of both sexes weighing 400 to 500 g were used for immunizations. Two guinea pigs were inoculated with each of the control or treated samples. All live virus control samples were initially inoculated intranasally, undiluted, and in 0.1-ml quantities. Each treated sample was inoculated intraperitoneally, undiluted, and in 0.2-ml quantities. On day 21, every animal received a booster 0.2-ml inoculation of a 1:10 dilution of the appropriate control or treated sample, and on day 28 the animals were bled. Equal quantities of sera from the two animals receiving the same inoculations were pooled and stored at −20 C until used in the HI tests. Control sera were obtained from uninoculated guinea pigs and animals inoculated with normal chicken allantoic fluid.

Calculations. For the purpose of comparison, a ratio was established by dividing the value obtained for a particular treatment method by the zero-hour control value. By definition, the zero-hour control always had the value of 1.00. For a particular time of a treatment, the average of the values of the four virus strains was calculated. Also, the average of all the time periods of each strain was determined; finally, the average of each time period for all four strains was calculated.

RESULTS

Effect of inactivation on infectivity. Results are shown in Table 1. The 18-, 48-, and 72-hr controls generally showed only a slightly lower infectivity when compared to the zero-hour controls. The length of treatment at 37 C did not seem to affect the titer. Formalin, Merthiolate, Merthiolate and Formalin, UV, and BPL significantly reduced infectivity. Formalin and Merthiolate and Formalin inactivation were complete by 18 hr. Merthiolate also rapidly inactivated influenza virus. However, with high-titering viruses, such as A/PR/8/34 ($EID_{50} = 10^{9.8}$), inactivation was slower, approximately 0.67 of the zero-hour control at 18 hr, 0.46 at 48 hr, and complete by 72 hr. Quantitatively, Merthiolate treatment alone seemed to be slightly less effective as compared to the other four methods. UV and BPL treatments completely inactivated all virus strains in 3 and 10 min, respectively.

Effect of inactivation on CCA. Examining the untreated and the BPL live virus-containing controls, the mean ratio for the four strains was very close to 1.0. However, with the exception of BPL treatment, the means of the experimental groups have values significantly higher than 1.0, with especially high values in the A2/Ga and B/Mass groups. It is to be noted that the BPL treatment markedly lowered the CCA values. In general, CCA values did not change significantly over an 18- to 72-hr period.

Effect of inactivation on HA. Control, Merthiolate, Formalin, Merthiolate and Formalin, and UV treatments apparently did not affect the HA values. However, it is to be noted that, as with CCA, BPL treatment lowered the HA activity considerably. There appeared to be no pattern of change with time.

Effect of inactivation on antigenicity. The values in Table 2 reflect the differences in the HI titer between animals inoculated with treated and zero-hour control samples. We realize that the immunization procedures for the live virus controls and the treated samples were different. However, no matter what procedure was used, the antigenicity of an inactivated virus preparation would probably be directly related to the antigen mass, and the antigenicity of a live virus would depend upon both the antigen mass and the ability of the virus to replicate in that animal host. These limitations must be considered when evaluating the results. The zero-hour control titers served as a reference point for the purposes of this comparison. Although many more tests would probably be required to determine whether titer differences were significant, certain trends were noticeable. Formalin, Merthiolate and Formalin, and UV treatment for 6 min appeared to reduce consistently the antigenicity of the A/PR/8/34 strain. Merthiolate, Merthiolate and Formalin, and BPL seemed to have a deleterious effect on the A2/Ga/1/67 strain, and BPL destroyed completely the antigenicity of the B/Mass/3/66 strain. The significance of this has yet to be determined.

DISCUSSION

Previous studies with viruses other than influenza revealed that BPL treatment did not affect HA or antigenicity (10). It was surprising that in our study BPL adversely affected the HA

TABLE 1. *Effect of Formalin, Merthiolate, β-propiolactone, and ultraviolet light upon infectivity, chicken cell agglutination, and hemagglutination of influenza virus*[a]

Treatment		Code[b]	Ratio of treated sample to zero-hour control														
			Infectivity[c]					CCA					HA				
Method	Time		A/PR/8	A2/Jap	A2/Ga	B/Mass	Avg[d]	A/PR/8	A2/Jap	A2/Ga	B/Mass	Avg[d]	A/PR/8	A2/Jap	A2/Ga	B/Mass	Avg[d]
Control, untreated	0 hr	C0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	18 hr	C18	0.95	0.93	0.83	0.83	0.89	0.73	0.71	1.85	1.10	1.10	1.00	1.00	1.00	2.00	1.25
	48 hr	C48	0.92	1.08	0.67	0.83	0.88	0.74	0.68	1.08	1.08	0.90	1.00	0.50	0.72	1.41	0.91
	72 hr	C72	0.87	0.88	Un	0.87	0.87[e]	0.45	0.84	1.85	1.45	1.15	1.00	0.50	0.72	2.00	1.06
Avg of C18, C48, C72			0.91	0.96	0.75	0.84	0.88	0.64	0.74	1.59	1.21	1.05	1.00	0.67	0.81	1.81	1.07
Formalin	18 hr	F18	0	0	0	0	0	0.88	0.91	1.85	1.95	1.40	1.00	0.50	2.00	1.41	1.23
	48 hr	F48	0	0	0	0	0	0.80	0.91	1.85	2.75	1.58	0.71	0.71	1.00	1.41	0.96
	72 hr	F72	0	0	0	0	0	1.23	0.95	1.23	2.00	1.35	0.71	0.71	1.00	1.41	0.96
Avg of F18, F48, F72			0	0	0	0	0	0.97	0.92	1.64	2.23	1.44	0.81	0.64	1.33	1.41	1.05
Merthiolate	18 hr	M18	0.67	0	0	0	0.17	0.63	1.13	1.77	1.61	1.29	1.00	0.71	1.40	1.00	1.03
	48 hr	M48	0.46	0	0	0	0.12	0.59	0.86	1.69	1.36	1.13	1.00	0.35	1.00	1.41	0.94
	72 hr	M72	0	0	0	0	0	0.61	1.00	1.69	2.11	1.36	1.00	0.71	1.00	0.50	0.80
Avg of M18, M48, M72			0.38	0	0	0	0.09	0.61	1.00	1.72	1.69	1.26	1.00	0.59	1.13	0.97	0.92
Merthiolate and Formalin	18 hr	MF18	0	0	0	0	0	0.76	1.05	1.00	2.26	1.27	1.00	0.71	0.50	2.00	1.05
	48 hr	MF48	0	0	0	0	0	0.84	1.05	1.93	1.43	1.31	0.71	0.71	1.00	1.41	0.96
	72 hr	MF72	0	0	0	0	0	0.94	1.10	1.54	1.89	1.37	0.50	0.71	1.00	1.41	0.91
Avg of MF18, MF48, MF72			0	0	0	0	0	0.85	1.07	1.49	1.86	1.32	0.74	0.71	0.83	1.61	0.97
Ultraviolet	3 min	UV3	0	0	0	0	0	1.06	1.16	0.69	1.89	1.20	2.00	0.71	0.50	1.00	1.05
	6 min	UV6	0	0	0	0	0	1.16	1.16	I	1.98	1.43[c]	1.41	0.50	0.50	1.41	0.96
Avg of UV3, UV6			0	0	0	0	0	1.11	1.16		1.94	1.32	1.71	0.61	0.50	1.21	1.01
β-Propiolactone																	
Control	10 min	BPLC	0.92	1.08	0.97	0.90	0.97	1.16	1.43	0.54	0.92	1.01	1.00	0.71	0.50	1.41	0.90
Treated	10 min	BPLT	0	0	0	0	0	0.50	I	I	0.70	0.60	0.35	0.18	<0.06	0.18	<0.19

[a] Abbreviations: CCA, chicken cell agglutination; HA, hemagglutination; A/PR/8, A/PR/8/34; A2/JAP, A2/Japan/170/62; A2/Ga, A2/Georgia/1/67; B/Mass, B/Mass/3/66; Un, unsatisfactory; I, indeterminate.

[b] The letter designations indicate treatment with the following: C, control; F, Formalin; M, Merthiolate; MF, Merthiolate and Formalin; UV, ultraviolet; BPL, β-propiolactone. The numbers following the letters indicate the length of treatment, in hours or minutes. For BPL, the letters C and T designate control and treated, respectively.

[c] Infectivity log ratio = $\log_{10}$ EID_{50} titer of sample/$\log_{10}$ EID_{50} titer of zero-hour control. Infectivity titers of the zero-hour controls of A/PR/8, A2/Jap, A2/Ga, and B/Mass were $10^{9.8}$, $10^{6.8}$, $10^{6.0}$, and $10^{7.8}$, respectively. Titers of $10^{0.5}$ to $10^{2.0}$ were regularly observed for most of the A/PR/8 inactivated samples and on several occasions for the other three virus strains. These low titers of HA activity in the allantoic fluids, which were shown to be due to the hemagglutinin contained in the inoculum and not to virus replication, were considered 0 for the purposes of determining the infectivity ratios.

[d] Average of ratios of the four strains.

[e] Average of ratios for A/PR/8, A2/Jap, and B/Mass strains.

TABLE 2. *Effect of Formalin, Merthiolate, β-propiolactone, and ultraviolet light on influenza virus antigenicity*

Treatment method and time (code)[a]	HI titer of sample compared with zero-hour control[b]				
	A/PR/8[c]	A2/Jap	A2/Ga	B/Mass	Avg[d]
C0	1.00	1.00	1.00	1.00	1.00
C18	0.75	1.25	1.17	6.00	2.29
C48	1.50	1.00	0.33	4.33	1.79
C72	1.50	0.83	0.17	4.67	1.79
(Avg)					1.72
F18	0.75	0.83	1.33	2.33	1.31
F48	0.58	1.17	1.08	2.00	1.21
F72	0.25	0.83	0.50	1.67	0.81
(Avg)					1.11
M18	0.83	2.00	1.16	6.00	2.50
M48	2.00	1.17	0.42	4.67	2.07
M72	2.33	0.83	0.33	12.00	3.87
(Avg)					2.81
MF18	0.17	1.13	0.33	0.67	0.58
MF48	0.33	3.00	0.75	2.00	1.52
MF72	0.13	4.08	0.25	0.67	1.28
(Avg)					1.13
UV3	2.33	1.00	0.42	6.00	2.44
UV6	0.33	2.00	0.83	2.33	1.37
(Avg)					1.91
BPLC	0.58	2.00	2.00	5.67	2.56
BPLT	0.58	0.50	0.33	0	0.35

[a] See footnote *b* of Table 1 for explanation of code.

[b] Antibody titer ratio = HI titer induced by immunization with sample/HI titer induced by inoculation of zero-hour control. The HI titers used to determine the antibody titer ratios were average values of three tests; the geometric mean antibody titers resulting from inoculation of the zero-hour controls of A/PR/8, A2/Jap, A2/Ga, and B/Mass were 512, 809, 51, and 40, respectively.

[c] Virus strain.

[d] Average of four strains.

and antigenic properties of influenza virus. In contrast, the other methods of inactivation (i.e., Formalin, Merthiolate, and UV light) resulted in an increase in HA and CCA activity and no apparent change in antigenicity. The more severe action of BPL was also reflected in its ability to reduce infectivity quickly and markedly.

In general, Merthiolate is used as a preservative in biological products. The fact that it can also inactivate viruses such as influenza has been noted previously (4). Our results corroborate these earlier findings.

Although there did not appear to be any strict relationship between HA and CCA values, nor between CCA value and antigenicity, the lack of sufficient testing precludes such a conclusion.

Considering the fact that the untreated controls contained live virus, our results showed that these samples demonstrate no greater HA or CCA content and antigenicity than the Formalin, Merthiolate, and UV-inactivated samples. Apparently, a period of inactivation greater than 18 hr was sufficient to reduce infectivity considerably without disturbing the antigenicity of the preparations.

The results of our antigenicity studies in guinea pigs revealed that one virus strain may react differently from another to a particular inactivating procedure. This would suggest that care must be exercised in attempting to apply conclusions obtained from the study of one virus group to another without experimentation. Since the antigenicity of BPL-treated influenza virus appears to be considerably reduced or destroyed, this method of treatment does not appear promising for influenza vaccine manufacture.

Our experiments revealed that combined treatment with Merthiolate and Formalin did not result in any greater loss in infectivity, nor any greater antigenicity, than treatment with either substance alone. Moreover, this combination of treatments may have reduced the antigenicity of two influenza strains as measured by HI antibody production in immunized guinea pigs.

With the development of newer methods of inactivating viruses for vaccine production, it is important to reevaluate the older methods. In this way, the technology for preparing killed vaccines can be updated and vaccines so produced can be improved.

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