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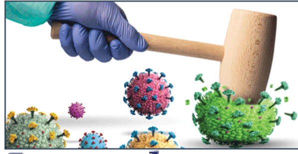

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THE REVERSAL *IN VIVO* BY BAL OF $HgCl_2$-INACTIVATED INFLUENZA A VIRUS IN THE CHICK EMBRYO*

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Using $HgCl_2$-inactivated influenza A virus (PR8 strain) Klein, Brewer, Pérez and Day (1) have shown that BAL can restore the activity of the virus *in vitro*. Employing white swiss mice as test animals, Klein and Pérez (2) were able to show that $HgCl_2$-inactivated virus could be completely reactivated *in vivo* when an intramuscular injection of BAL preceded the intranasal instillation of the inactivated virus. However when BAL was injected 30 minutes *after* the intranasal instillation of the inactivated virus, no reactivation was obtained. By contrast, $HgCl_2$-inactivated pneumococci could be reactivated *in vivo* when BAL was injected both before and after the inactivated bacteria. The results therefore suggested that influenza virus when adsorbed onto its host cell was removed from the action of a chemical agent such as BAL.

The following study was undertaken to determine whether by employing the BAL-$HgCl_2$ system, reactivation of virus could be obtained in the chick embryo and, if so, under what conditions such reversal would occur.

METHODS AND MATERIALS

The PR8 strain of influenza A virus grown in 10-day old chick embryos was used throughout this study. For detection of virus, allantoic fluids were tested with a fresh saline suspension of thrice-washed chicken red cells. Four tenths ml of each fluid was placed in a Wassermann tube and 0.4 ml of a 1 per cent chick red cell suspension added. The reaction was read after 75 minutes at room temperature. For quantitative titration of fluids the Salk pattern test (3) was used and the titers obtained ranged from 1:256 to 1:4096. A sterility test was performed each time the stock virus was used.

Inactivation and Toxicity Tests. A number of assays were performed to determine the minimal concentration of aqueous $HgCl_2$ which would completely inactivate a 10^{-4} dilution of the virus in broth. Inactivated virus was inoculated into groups of 10-day chick embryos and the presence of virus determined after 42 hours incubation, by hemagglutination. It was found that 1:20,000 $HgCl_2$ would completely inactivate the virus in 30 minutes at room temperature (28 C) and this concentration was used in all experiments.

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Several assays were done to determine the maximal concentration of freshly diluted aqueous BAL which could be given by the allantoic route. It was found that 0.1 ml of a 1:200 aqueous dilution of BAL killed 25–60 per cent of the embryos. However, 0.1 ml of a 1:400 aqueous dilution was non-lethal and was used as the reactivating dose.

Reactivation Technique. Stock allantoic fluid virus was diluted to 10^{-4} in Heart Infusion Broth, pH 7.4, and inactivated by contact with an equal volume of 1:20,000 aqueous $HgCl_2$ for a period of 30 minutes at room temperature. A group of embryos received 0.1 ml of inactivated virus by the allantoic route. Of these, a number were kept as controls for inactivation while other groups received 0.1 ml. of 1:400 aqueous BAL at different intervals either before or after the injection of inactivated virus. Active virus was diluted to the same final concentration as the inactivated virus and was left at room temperature for 30 minutes before inoculating each of a group of embryos with 0.1 ml. All embryos were incubated for 42 hours at 37 C, left overnight at 4 C, and the fluids harvested and tested for presence of virus.

Mouse Tests. Infectivity of allantoic fluids for mice was determined in some instances as a check on the hemagglutination titration. Groups of 4 to 6 mice were used for each dilution and 0.05 ml was instilled intranasally under light ether anesthesia. The mice were kept for 9 to 10 days; all surviving mice were sacrificed by heavy ether anesthesia and the lungs observed for the degree of pulmonary consolidation.

EXPERIMENTAL

Several experiments were performed to determine *in vitro* the action of BAL in restoring the activity of the virus as measured by infectivity for chick embryos. Infected allantoic fluid was diluted to 10^{-4} in broth and 10 ml of this dilution was thoroughly mixed with 10 ml of 1:20,000 aqueous $HgCl_2$ and left at room temperature. At various time intervals, 2 ml of the mixture was removed and placed in contact with an equal volume of 1:1000 aqueous BAL for 15 minutes at room temperature. After this period 0.1 ml of the reactivation mixture (virus plus $HgCl_2$ plus BAL) was injected by the allantoic route into 6–8, 10-day chick embryos. A number of eggs received inactivated virus at the end of each inactivation period. A control group of eggs received 0.1 ml of active virus diluted to the same final concentration as the inactivated virus and kept at room temperature for 2 hours. Table I shows the results of a typical assay.

From table I it can be seen that when BAL was added to virus which had been inactivated by 30 minutes contact with $HgCl_2$, the virus was reactivated, as shown by the fact that all embroys became infected. Even after 1 hour contact with $HgCl_2$ the addition of BAL resulted in a considerable degree of reactivation, 5 out of 8 embryos becoming infected with virus. After 2 hours contact of the virus with the $HgCl_2$, the BAL could still effectively reactivate the virus. However, after 5 hours contact no reversal could be obtained.

In order to determine whether the reactivation obtained after 30 minutes contact of virus with $HgCl_2$ was complete the following experiment was carried out.

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The virus was diluted to 10^{-9} in broth and dilutions 10^{-6} to 10^{-9} were inactivated by contact with 1:20,000 $HgCl_2$ for 30 minutes. Two ml of the inactivated virus were mixed with 2 ml of 1:1000 aqueous BAL and the BAL left to act for 15 minutes. Reactivation was determined by the inoculation of 0.1 ml of each mixture into groups of 6–8 embryos. Table II shows the results of this test. The data summarized in table II show that after 30 minutes contact with $HgCl_2$ only partial reactivation could be obtained with virus diluted to 10^{-7}, and none beyond that point.

In vivo reactivation by BAL. Before carrying out the studies on the *in vivo* reactivation of $HgCl_2$ inactivated virus, the possibility of action by BAL on the multiplication of the active virus in the embryo was explored.

TABLE I

Effect of time of contact with $HgCl_2$ on in vitro *reactivation of influenza A virus by BAL*

$HgCl_2$ INACTIVATION PERIOD	BAL ADDED TO MIXTURE AFTER INACTIVATION PERIOD	INACTIVATION CONTROLS	VIRUS CONTROL
min.			
30	7/7*	0/6	
45	6/7	0/5	
60	5/8	0/6	
120	3/7	0/6	4/5
300	0/8	0/5	

* In this and the succeeding tables, the numerator is the number of embryos positive for virus and the denominator the number of surviving embryos of initial test group. Only fluids from *living embryos* after 42 hrs. incubation are recorded.

TABLE II

Effect of dilution of influenza A virus on in vitro *reactivation*

DILUTION OF VIRUS	ACTIVE VIRUS	$HgCl_2$-INACTIVATED VIRUS	INACTIVATED VIRUS PLUS BAL
10^{-6}	6/6	0/6	3/6
10^{-7}	4/6	0/7	2/5
10^{-8}	4/4	0/6	0/8
10^{-9}	2/6	0/6	0/6

Sixty embryos received 0.1 ml of a 10^{-4} broth dilution of the stock virus. The embryos were divided into groups of 10. Single groups received 0.1 ml of 1:400 aqueous BAL at different time intervals (15, 30, 60, 120 and 300 minutes) *after* the injection of active virus. A virus control group was run as usual. The results showed that the BAL had no detectable inhibitory action on the virus since all embryos became infected and the allantoic fluids showed high individual hemagglutinin titers. These results are therefore similar to those obtained with BAL in mice (2).

The degree of reversal obtained when BAL was given at increasing time intervals *before* the injection of inactivated virus is shown in table III. As can be seen when a single injection of BAL was given either 5 or 7 hours before the

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inactivated virus, no reactivation was obtained. When the BAL was given at increasingly shorter time intervals, i. e. from 3 hours to 0.25 hour before the inactivated virus, the degree of reactivation increased as the time intervals shortened. It can be seen that reactivation (100 per cent infectivity) was obtained when BAL was given either at 0.5 or 0.25 hour before the inactivated virus. The high degree of reactivation obtained at the one-hour interval suggests that BAL keeps its full activity for at least one hour after injection into the allantoic cavity.

Action of BAL injected after inactivated virus in vivo. The degree of reversal obtained when BAL was given *in vivo* after the inactivated virus was studied in

TABLE III

In vivo *reversal by BAL injected* before *inactivated virus*

EXP. NO.	BAL INJECTED AT INTERVALS OF:*						INACTIVATION CONTROL	VIRUS CONTROL
	7 hr.	5 hr.	3 hr.	1 hr.	0.5 hr.	0.25 hr.		
1	0/7	0/7	1/9	6/7	9/9	8/8	0/10	6/7
2	—	0/8	4/8	7/7	7/7	7/7	0/8	7/7

* Before inoculation of the inactive virus, embryos from each group received a single injection of 0.1 ml of 1:400 aqueous BAL at one of the indicated time intervals.

TABLE IV

In vivo *reversal by BAL injected* after *inactivated virus*

EXP. NO.	BAL INJECTED AT INTERVALS OF:				INACTIVATION CONTROLS	VIRUS CONTROLS
	15 min.	30 min.	60 min.	120 min.		
1*	5/12	4/11	0/11	0/10	0/11	10/10
2*†	7/8	4/6	3/8	0/7	0/10	6/6
3*†	5/7	3/7	2/4	0/7	0/10	6/6
4†	7/8	2/5	7/8	2/7	0/10	5/7
5	9/10	3/9	2/10	2/9	0/9	8/8
6	7/10	8/11	3/9	4/11	0/12	11/11

* Fluids tested for presence of active virus by mouse inoculation.

† Test for presence of free-inactivated virus (see table V).

some detail. The results obtained when a single dose of 0.1 ml of 1:400 aqueous BAL was given at varying intervals of time are shown in table IV. Though the results varied slightly, it can be seen that the degree of reactivation diminished progressively as the time intervals between the injection of virus and that of BAL were increased. Optimum reactivation was obtained when BAL was given 15 minutes after the inactivated virus. (Preliminary experiments showed that essentially the same degree of reactivation was obtained whether BAL was given either 5 or 15 minutes after the inactivated virus and the longer interval was chosen for convenience.)

In experiments 1 to 3 no reactivation was obtained when BAL was given 120 minutes after the injection of inactivated virus. In fact, in experiment 1, no re-

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activation was observed when BAL was given at the 60 minute interval. On the other hand in experiment 4 a greater degree of reactivation, as shown by the proportion of infected to non-infected eggs, was observed when BAL was injected 60 minutes after virus than when BAL was injected 30 minutes after the inactivated virus.

Presence of free $HgCl_2$-inactivated virus in the allantoic cavity. If the $HgCl_2$-inactivated virus were completely adsorbed by its host cells prior to the inoculation of BAL the results would indicate that a chemical agent such as BAL could act on influenza virus following its adsorption onto the host cells. It was obvious however that some free unadsorbed virus might account for the reversal obtained when BAL followed the virus. The experiments to determine this point formed

TABLE V

Presence of free inactivated virus in the allantoic cavity

EXPERIMENT NO.†	ALLANTOIC FLUID HARVESTED AFTER INJECTION OF INACTIVE VIRUS AT:*			
	15 min.	30 min.	60 min.	120 min.
2	1/7(1:1024)‡	0/7	0/8	0/7
3	0/5	0/6	0/6	0/6
4	2/7(1:2048) (1:512)	0/6	1/8(1:512)	0/6
	Controls§			
2	0/7	0/6	0/7	0/7
3	0/5	0/6	0/5	0/6
4	0/7	0/8	0/8	0/8

* Aqueous BAL added to allantoic fluid after removal. After 15 min. contact 0.5 ml injected per embryo.

† Numbers correspond to experiments in table IV.

‡ Titer of positive fluids by Salk pattern test.

§ Water added to allantoic fluid after removal. After 15 min. contact 0.5 ml injected per embryo.

an integral part of experiments 2, 3 and 4 shown in table IV. The technique employed was as follows: Allantoic fluid from eggs that had previously received 0.1 ml of inactivated virus (10^{-4} dilution) was removed at 15, 30, 60 and 120 minutes *after* the injection of inactivated virus. Fluid from three embryos was removed at each time. The fluids from the three embryos were pooled and placed in the icebox at once. A portion of each pool was mixed with 1:1000 aqueous BAL and another portion with sterile distilled water. After 15 minutes at room temperature 0.5 ml of the mixture was injected into eight 10-day chick embryos which were then incubated for 42 hours and tested for the presence of virus. The results obtained by employing this procedure are shown in table V.

As can be seen from the proportion of infected to non-infected eggs shown in table V, the addition of BAL *in vitro* to allantoic fluids indicated the presence of some free, inactivated virus in the fluids in two experiments. The control in-

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jections of the pooled fluids were negative in all instances as shown in table V a fact which indicated that no active virus was present in the $HgCl_2$-treated virus. The significance of these findings in relation to the *in vivo* reactivation of the virus at the various time intervals will be discussed below.

Mouse tests. It has been shown (4, 5) that absence of hemagglutinating activity in allantoic fluids does not necessarily mean that the fluid contains no active virus. In fact, allantoic fluids have been shown to contain as much as 10,000 mouse ID_{50} doses of virus without revealing any hemagglutinating activity (5).

TABLE VI

Effect of dilution of virus on reversal by BAL in vivo*

EXPERIMENTAL GROUP	DILUTIONS OF VIRUS				
	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
#1					
Active Virus	—	6/6	6/6	6/7	7/8
Inactivated Virus	—	0/5	0/5	0/6	0/6
BAL injected 15 min. after inactivated virus	—	6/6	6/7	7/8	4/5
#2					
Active Virus	—	—	7/8	9/10	9/10
Inactivated Virus	—	—	0/7	0/8	0/9
BAL injected 15 min. after inactivated virus	—	—	0/6	0/8	0/8
#3					
Active Virus	—	8/8	7/7	4/7	—
Inactivated Virus	—	0/8	0/8	0/8	—
BAL injected 15 min. after inactivated virus	—	0/6	0/7	0/7	—
#4					
Active Virus	6/6	6/6	6/6	6/6	—
Inactivated Virus	0/6	0/6	0/6	0/6	—
BAL injected 15 min. after inactivated virus	9/10	6/7	5/9	2/8	—

Experiments 1, 3: 8 embryos per experimental group.

Experiment 2: 10 embryos per experimental group.

Experiment 4: 6 embryos per experimental group (except in reactivation groups where 10 embryos per group were used).

* Decimal dilutions of virus inactivated by 30-minute contact with 1:20,000 aqueous $HgCl_2$ at room temperature; 0.1 ml of corresponding decimal dilution of inactivated virus injected into a group of 12–20 embryos *half* of which were injected with 0.1 ml 1:400 aqueous BAL 15 min. later; one-half of each group kept as inactivation controls; 0.1 ml of corresponding decimal dilution of active virus (after 1:1 dilution in saline) injected into virus control (active virus) groups.

The possibility was therefore considered that fluids that did not show hemagglutinating activity in the above experiments might nevertheless contain a small amount of active virus. Accordingly, pools of allantoic fluids from *each* experimental group in experiments 1, 2 and 3 of table IV, were tested for infectivity in white mice as outlined under "Methods". In every case allantoic fluid which did not agglutinate chicken red cells did not infect mice. Allantoic fluids having high hemagglutinin titers, were highly infectious for mice. The infectivity endpoints of these fluids ranged between 10^{-5} to 10^{-7}.

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In another series of tests, allantoic fluids withdrawn from eggs at various intervals after receiving the usual dose of inactivated virus (0.1 ml of 10^{-4} dilution) were tested for mouse infectivity. In no instance was the presence of active virus in the allantoic fluid revealed by these tests.

The degree of reversal by BAL. A group of experiments was carried out in order to determine quantitatively the degree of reversal obtained. The results obtained are given in table VI. It can be seen that the results were not consistent. In experiment 1 the virus was reactivated to its 50 per cent infectivity-endpoint (10^{-9}) while in other experiments no reactivation was obtained even when the virus was diluted to only 10^{-6}. These discrepancies have not been satisfactorily explained.

Reactivation by varying concentrations of BAL. The effect of varying the concentrations of BAL used for *in vivo* reactivation is shown in table VII. Starting with an aqueous dilution of 1:200 BAL, varying dilutions were given to different

TABLE VII

*Effect of varying concentrations of BAL on reactivation**

EXPERIMENT	CONCENTRATION OF AQUEOUS BAL USED (0.1 ML INJECTED)							INACTIVATION CONTROL	VIRUS CONTROL
	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12,800		
1	6/7†	8/9	9/12	10/11	5/10	0/12	0/11	0/10	7/7
2	—	—	10/12	6/10	4/9	1/10	0/10	0/10	8/8

* 10^{-4} virus in broth inactivated by contact with 1:20,000 aqueous $HgCl_2$ for 30 min. at room temperature. 0.1 ml of virus thus inactivated injected into embryos; 12 embryos left as inactivation controls; 0.1 ml aqueous BAL in the various concentrations used given *in vivo* 15 min. after inactivated virus; 0.1 ml of 10^{-4} virus in broth diluted 1:2 in water, given to virus controls; 12 embryos per experimental group except virus control which had 8 embryos.

† 5 embryos dead.

groups of embryos 15 minutes *after* each embryo had received 0.1 ml of inactivated virus. The reactivating dose (0.1 ml) was the same for all dilutions used. It can be seen that Bal was still effective when used in a dilution of 1:3,200 in aqueous solution. The proportion of infected to non-infected embryos shows that BAL was very effective in reactivating the virus when used in dilutions of 1:400 to 1:1,600. Within the conditions of the experiment a dilution of 1:3,200 seems to be the effective endpoint for obtaining reversal.

DISCUSSION

Our results demonstrate that the infectivity of the PR8 virus inactivated by contact with $HgCl_2$ can be restored by the intra-allantoic injection of BAL either before or after the inactivated virus. The results are in general similar to those previously obtained in mice though the time range within which BAL can be given *in vivo* to get effective reactivation is greater in the chick embryo than in the mouse. This is so whether the BAL is given before or after the inactivated virus. For obtaining reactivation in the mouse the BAL had to be injected no

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more than either one hour before or 30 minutes after the intranasal instillation of inactivated virus. In the studies with the chick embryo on the other hand, reversal of inactivated virus could be obtained when BAL was given *in vivo* as long as three hours before or two hours after the injection of the inactivated virus. In the mouse, however, complete reactivation was always obtained when BAL was given five minutes before the virus, while complete reactivation was not regularly obtained in the chick embryo.

Though we have no direct evidence that the $HgCl_2$–inactivated virus becomes adsorbed onto the cells of the allantoic sac, adsorption probably occurs in view of the following facts. Active and ultraviolet-irradiated (inactive) influenza A virus (4, 6) have been shown to become adsorbed on living cells of the allantoic sac, and virus inactivated by $HgCl_2$ has been shown to be readily adsorbed onto chicken red cells (2). It is probable therefore that much of the $HgCl_2$–inactivated virus is adsorbed onto the cells of the allantoic sac. Our recovery of only a small amount of free inactivated virus in the allantoic fluid is in keeping with these observations.

It was of particular interest to determine whether a chemical agent such as BAL could reach the virus after adsorption onto the cells of the allantoic sac. Although reversal was in fact obtained when BAL was injected into the allantoic cavity two hours after the inactivated virus, the demonstration of free inactivated virus in some experiments does not permit the conclusion that we are dealing with the action of a chemical agent directly on adsorbed or intracellular virus. The reversal obtained with BAL may result from the action of the compound on both free and adsorbed virus or on free virus alone.

SUMMARY

A study was made of conditions under which reactivation of $HgCl_2$-inactivated influenza A virus (PR8 strain) would ocur in the chick embryo by the use of aqueous BAL. The following results were obtained:

1. A 10^{-4} dilution of allantoic fluid virus in broth was completely inactivated by 1:20,000 aqueous $HgCl_2$ after contact for 30 minutes at room temperature (28 C).

2. The virus-Hg complex did not dissociate upon dilution in the allantoic fluid as shown by lack of infectiousness of the fluid for mice and chick embryos after injection of inactivated virus.

3. Aqueous BAL injected via the allantoic route in 1:400 dilution could reactivate the virus when given from 3 to 5 hours *before* and from 0.25 to 2 hours *after* the injection of inactivated virus.

4. Following the inoculation of inactivated virus into the allantoic cavity some free unadsorbed virus was demonstrated in the fluid in some experiments. It was therefore not possible to determine whether reactivation resulted from the action of BAL on the adsorbed virus.

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