

On the Preparation of Purified Influenza Virus Vaccine

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In 1933 Smith, Andrewes and Laidlaw (1), by utilizing a technique in experimental animals previously found effective in canine distemper experiments, reported on the action of a filterable virus recovered from epidemic influenza patients in Great Britain. Although virus etiology of influenza had been dealt with previously by other investigators, the experiments of Smith, Andrewes and Laidlaw utilizing ferrets were so convincing and definite that great interest was at once awakened in this work. These British authors for the first time demonstrated that nasal washings from patients ill with epidemic influenza contained a virus which was pathogenic on intranasal inoculation of ferrets. These animals exhibited characteristic temperature responses and presented evidence of infection in the nasal sinuses and the lungs. Repeated animal passage from one ferret to another resulted in enhanced virulence of the newly discovered epidemic influenza virus for ferret lungs, and gradually a more pronounced pneumonia began to appear in virus passage animals.

Following these first clear-cut experiments dealing with the isolation of epidemic influenza virus and its transmission in ferrets, various immunological aspects of the virus were determined. It was found that an animal convalescing from virus influenza exhibited a strong degree of resistance against further infection during a period of several months. Also it was observed that blood serum recovered from convalescent animals brought about neutralization of fully virulent epidemic influenza virus.

The knowledge of influenza virus was soon broadened by demonstration that the virus is pathogenic for Swiss mice (2) and, still later, that it is able to infect chick embryo tissues (3), and also bring about hemagglutination of erythrocytes of different species of animals (4). These two latter methods of infection along with hemagglutinating action (see Table 1) soon expedited widespread study of new strains of influenza virus, and many laboratories in different parts of the world, including Great Britain, the United States, Russia and Australia, began to publish reports dealing with different properties of influenza virus as isolated in different places. Immunization experiments in mice, ferrets, and human beings dealing with different strains of the virus were reported, and in the course of these it became apparent that differences existed between different strains. In other words, types of epidemic influenza virus were established (5). At the present time these types include type A and type B strains (see Table 2). Viruses of each of these types produce specific infections, and there is practically no crossover immunity between the two types. Possibly further types are yet to be discovered; however, present efforts in connection with the specific prevention of influenza utilize only well known strains of types A and B virus.

Experience in the serology of influenza soon shows the utility of the hemagglutination test. This test is a useful indicator of both the lethal potency of freshly prepared virus for Swiss mice and action of such virus when made into vaccine. Also the degree of neutralization of the hemagglutinating properties of the virus by serum serves as a rapid indicator of potency of influenza antibody, and resistance of experimental animals and human beings.

Prophylactic Influenza Antiviral Serum

During the last three or four years, considerable work has been done on influenza antiviral serum (6). No doubt the initial meager results obtained with the early vaccines prompted the preparation of influenza antiviral serum of such nature that this might be used as an intranasal prophylactic in case influenza became imminent. As yet there appears to be little controled evidence, except that from Russian laboratories, which is available for judging the efficacy of influenza antiviral serum. However, some recent evidence has appeared indicating utility of influenza virus vaccine prepared as described below.

Influenza Virus Preparation on a Large Scale

Usually ten-day incubated hen eggs are used for preparation of influenza virus in large quantities for making purified and concentrated vaccine. Actual inoculation of these eggs comprises first treating the shell with a suitable antiseptic. We paint an area on the large end of each egg with Tincture of "Merthiolate" (Sodium Ethyl Mercuri Thio-salicylate, Lilly). Following this, a puncture hole is made in this area of the egg over the air cell. Using an automatically filling hypodermic syringe connected with a reservoir of "seed" virus, 0.2 cc. injections are made into the embryonic fluids of the egg using for the inoculum type A virus diluted 1:10,000 to 1:50,000 or more and type B virus diluted 1:1,000 to 1:5,000 or more (see Fig. 2). The openings in the shell are then sealed with paraffin, and the eggs are reincubated for approximately 48 hours. At this time the eggs are removed from the incubator and treated with antiseptic, following which a portion of the shell covering the air cell is removed aseptically. At this point the membranes surrounding the embryo are ruptured under sterile precautions, and the larger blood vessels are broken to assure as much hemorrhage as possible. (In this way the red blood cells of the embryo suffice for adsorption and subsequent elution of virus.) The mixture of virus-containing embryonic fluids and blood is then harvested by means of suitable aspiration apparatus and slight negative pressure. This material when accumulated in one-pint amounts is rapidly chilled at 2° to 50° C. in a water bath, and allowed to stand at this temperature for two hours or longer. Under these conditions the virus brings about cold hemagglutination of the red corpuscles and is adsorbed on the corpuscles in this process. When this has taken place, low speed centrifugation is done, and the supernatant fluid while cold is removed aseptically and discarded. Usually by far the greater proportion of the virus has been adsorbed by the red blood corpuscles, and relatively little is lost in the supernatant fluid.

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The sedimented red blood corpuscles containing adsorbed influenza virus are resuspended in fresh physiological salt solution, preferably in one-tenth the volume of the original embryonic fluid, and are then subjected to a temperature of 37° C. for three to four hours. When treated in this way the cells redisperse from their previous agglutinated condition, and the virus elutes from the cells into the saline. When this reaction is complete, low speed centrifugation is again done, and the supernatant fluid containing the major portion of the original virus is aspirated off the red blood cell sediment (see Table 3 for hemagglutinating potency).

Conversion of Influenza Virus Into Vaccine

This purified virus concentrated by adsorption and elution as described is accumulated in large volumes and is inactivated with formalin 1:2,000 at ice box temperature. "Merthiolate," previously found satisfactory by the present author (7) for this purpose, is added in a 1:40,000 concentration as a preservative, and type A and type B vaccine lots are pooled in equal quantities. Epidemic influenza vaccine prepared in this way is subjected to immunization tests in Swiss mice. In these tests two doses of 0.01 cc. of vaccine each diluted up to 1.0 cc. in volume and given intraperitoneally must immunize against 10,000 LD50 of type A virus, and two doses of 0.0001 cc. of vaccine similarly diluted must immunize against 1,000 LD50 of type B virus (see Table 4 for immunizing potency in mice). Both type A and type B challenge virus comprise mouse lung passage material diluted decimally and given intranasally. The smaller challenge dose of type B virus is made necessary by the lower virulence of this virus; however, this is compensated for through immunization with much smaller doses of vaccine. The usual safety and sterility tests prescribed by the National Institute of Health finally are conducted, and upon release by the Institute the vaccine is ready for dispensing.

Responses to Epidemic Influenza Virus and Vaccine

In the course of earlier experiments with influenza virus and vaccine in ferrets, Swiss mice, and human beings, it was observed that various kinds of specific responses were produced against these agents. Serological studies of both animal and human blood before and after influenza and before and after use of influenza virus vaccine have shown marked differences in complement fixing titer, virus neutralizing titer, and protective antibody titer. Thus far, however, it has been impossible to utilize any definite titer of any particular antibody (i.e., comparable to the Schick test level of antitoxin in diphtheria immunity) to define a state of immunity against actual influenzal disease. In general, however, the lower antibody titers are associated with susceptibility, while the higher antibody titers are associated with resistance. The rather regular increase in antibody content during the course of infection has in fact some diagnostic significance in proving or disproving influenza, especially in view of the difficulties of diagnosing influenza by clinical grounds

alone. In other words, if serum drawn in the acute stage of an influenza-like condition is compared with serum drawn in early convalescence and the "convalescent" serum is found to contain from four to eight or more times as potent antibodies as the "acute" serum, the specific diagnosis may be made more certain.

Immunity Against the Disease

In view of the foregoing facts concerning the serology and immunology of influenza virus, it is finally necessary to observe the practical results obtained in human beings by use of influenza virus vaccine, in order to judge efficacy of this biological. During the widespread but rather mild epidemic of influenza in November and December, 1943, it was possible to do this in a limited way, and a military commission has reported favorable results in approximately 12,000 subjects comprising certain Army ASTP personnel (8). About half of these were given one dose of influenza virus vaccine types A and B prepared as has been described, while the other half were given saline as a control. Both treatments comprised a one-dose hypodermic injection of 1 cc. It was observed that the incidence of clinical influenza was markedly lowered in the immunized group as compared to the control group, the actual figures being about one case in the immunized to 3.2 cases in the non-immunized. This practical showing against the natural disease as it appeared last year, together with good immunizing capacity of purified and concentrated influenza virus vaccine in human volunteers who later received active virus by nasal spray, have increased interest in further use of such vaccine in advance of the next epidemic of influenza. The type or types of influenza virus which may cause the next epidemic are of course unpredictable.

Summary

1. The main properties of epidemic influenza virus have been reviewed.
2. Influenza antiserum and its possible limited use in a prophylactic way have been referred to.
3. Preparation of influenza virus on a large scale and its conversion into vaccine have been described stepwise.
4. Immunizing action of purified influenza virus vaccine, types A and B, as prepared on a large scale, has been shown experimentally by results obtained in Swiss mice.

References

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Table 1. Hemagglutination Test of Influenza Virus

Final virus dilution	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
Virus dose	(1-4) 1.0 cc.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
2% Chicken red blood cells	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Room Temperature One Hour										
PR8A Virus	+++++	+++++	+++++	+++++	+++	++	+	0	0	0
Weiss A Virus	+++++	+++++	+++++	+++++	+++	+	0	0	0	0
Lee B Virus	+++++	+++++	+++++	+++++	+++	+	0	0	0	0

Table 2. Hemagglutination Test of Influenza Virus Strains to Check Strain type.

Final dilution of serum	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
Serum dose	(1:2.5) 0.5 cc.	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Virus dose*	0.5 cc.	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

Room Temperature Five Minutes

2% Chicken R.B.C.	1.0 cc.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
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Room Temperature One Hour

PR8A Chicken Anti-serum†	0	0	0	0	0	+	+++	+++	+++	PR8A virus†
Weiss A Chicken Anti-serum	0	0	0	0	0	+	+++	+++	+++	Weiss A virus
Lee B Chicken Anti-serum	+++	+++	+++	+++	+++	+++	+++	+++	+++	Lee B virus
PR8A Chicken Anti-serum	0	0	+	+	+	+	+++	+++	+++	PR8A virus
Weiss A Chicken Anti-serum	0	0	0	0	+	+	+++	+++	+++	Weiss A virus
Lee B Chicken Anti-serum	+++	+++	+++	+++	+++	+++	+++	+++	+++	Lee B virus
PR8A Chicken Anti-serum	+++	+++	+++	+++	+++	+++	+++	+++	+++	PR8A virus
Weiss A Chicken Anti-serum	++	+++	+++	+++	+++	+++	+++	+++	+++	Weiss A virus
Lee B Chicken Anti-serum	0	0	0	0	0	+	++	+++	+++	Lee B virus

* Virus dose was eight 50 per cent hemagglutinating units.

† Specific chicken antiserum prepared by injecting chickens with two intraperitoneal doses of 5 cc. of virus, with an interval of a week between the doses, then bleeding the chickens a week after the last dose.

‡ The three viruses at different passages were assigned three section code numbers. The first or F section denotes the number of ferret passages; the second or M section denotes the number of mouse passages; the third or E section denotes the number of egg passages. In this experiment we used PR8A virus F198-M593-E14; Weiss A virus F3-M32-F21; and Lee B virus F8-M137-E58.

Table 3. Hemagglutination Test of Concentrated Influenza Lee Type B Virus Lot No. 734

Final virus dilution	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
Virus dose	(1-4) 1.0 cc.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
2% Chicken red blood cells	1.0 cc.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Concentrated virus	++++	++++	++++	++++	++++	++++	++++	++	+	0
Supernatant discard	++++	++++	++	+	+	0	0	0	0	0

Table 4. Mouse Immunity Test Protocol of Influenza Virus Vaccine

PR8A virus (mouse-lung suspension)*	10-1	10-2	10-3	10-4	10-5	10-6	10-7
Mice immunized with 10-2 dil. of vaccine	5, 5, 5 S, S, S	6, 7, S S, S, S	S, S, S S, S, S	7, 8, S S, S, S	0	0	0
Non-immunized mice	0	0	0	5, 5, 5 6, 6, S	6, 6, 6 7, 7, 9	8, 8, 8 10, S, S	8, S, S S, S, S
Weiss A virus (mouse-lung suspension)*	10-1	10-2	10-3	10-4	10-5	10-6	10-7
Mice immunized with 10-2 dil. of vaccine	6, S, S †, S, S	S, S, S S, S, S	5, S, S S, S, S	S, S, S S, S, S	0	0	0
Non-immunized mice	0	0	0	6, 6, 7 7, 8, 9	7, 8, 8 9, 9, S	9, 9, S S, S, S	8, 9, S S, S, S
Lee B virus (mouse-lung suspension)*	10-1	10-2	10-3	10-4	10-5	10-6	10-7
Mice immunized with 10-4 dil. of vaccine	8, S, S S, S, S	S, S, S S, S, S	S, S, S S, S, S	S, S, S S, S, S	0		
Non-immunized mice	0	5, 7, 8 9, 9, S	5, 8, 9 9, S, S	6, 9, 10 S, S, S	S, S, S S, S, S		

Number indicates day of death of individual mouse.

"S" indicates survival ten days.

* The three viruses, as mouse-lung suspensions in dilutions indicated, were given intranasally.

† One mouse killed accidentally.