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Host defence mechanisms against influenza infection. II. Protection of mice with vaccines against A/Port Chalmers/1/73 and B/Hong Kong/5/72

F. A. ENNIS
M.D., M.A.

M. A. WELLS
B.A.

D. W. BARRY
M.D.

S. DANIEL

J. MANISCHEWITZ
M S.

Department of Health, Education and Welfare, Public Health Service, Food and Drug Administration, Bureau of Biologics, Division of Virology, Bethesda, Maryland 20014

Summary

Influenza virus strains A2/Port Chalmers/1/73 (H_3N_2) and B/Hong Kong/5/72 were adapted to mice by serial passage of virus infected mouse lungs. The adapted viruses were used to challenge mice after they had been immunized with serial dilutions of several bivalent vaccines, containing both A/Port Chalmers and B/Hong Kong antigens. Vaccines included both whole virus and split-product preparations disrupted by Tween 80 and ether (ET) or tri-N-butyl phosphate (TBP).

These bivalent vaccines induced antibodies and conferred protection to mice against challenge with approximately 30 LD_{50} of the adapted A2/Port Chalmers virus. Significant protection against lethality ($P<0{\cdot}01$) was observed in groups of mice which developed haemagglutinin-inhibition antibody titres of $\geqslant 1:16$ following immunization. Protection was not statistically significant in groups of mice which developed antibody titres of $\leqslant 1:8$ to the A/Port Chalmers/1/73 virus following immunization with higher dilutions of certain vaccines.

In a similar fashion, current bivalent vaccines containing B/Hong Kong/5/72 stimulated antibodies and conferred significant protection to immunized mice against lethal challenge with the adapted B/Hong Kong virus. Protection was demonstrated against approximately 30 LD_{50} of adapted virus in groups of mice which developed mean antibodies of $\geqslant 1:16$. Groups of mice, which developed antibody titres of $<1:8$ following immunization with certain dilutions of these vaccines, were not significantly protected. These studies confirm and extend observations made with formerly circulating influenza viruses, i.e. immunization with a specific inactivated vaccine in adequate dosage confers protection against a challenge dose of virus lethal to unimmunized mice.

Correspondence: Dr F. A. Ennis, Division of Virology, Bureau of Biologics, Department of Health, Education and Welfare, Bethesda, Maryland 20014, U.S.A.

Introduction

The immune response to influenza vaccines and subsequent protection against virus challenge have been reported in many animal species. Most of the studies were performed many years ago, when Francis (1939), Fazekas de St Groth and Donnelly (1950) and others observed that inactivated influenza A viruses conferred specific protection to mice subsequently challenged with mouse-adapted influenza virus. Kaye, Dowdle and McQueen (1969) later showed some degree of cross-protection between members of the H_2N_2 subtype of influenza A virus, when mice were immunized with an inactivated vaccine and then were challenged with mouse-adapted strains of various H_2N_2 influenza A viruses derived from strains which circulated between 1957 and 1965. These studies indicated that influenza A viruses, inactivated by formaldehyde, and given in adequate amounts, would produce an immune response as measured by serum haemagglutination-inhibiting antibodies and by protection against subsequent lethal challenge with the mouse-adapted virus of the same or a closely related strain. Although other animal models have been used to study the immune response to influenza vaccines and infection, the mouse model of pulmonary infection has been used most extensively.

The purpose of the studies described here was two-fold: (1) there have been no reports of viral challenge studies in animal models using the recently developed split-product influenza vaccines which have been licensed in the United States. These vaccines are made by using chemicals which disrupt the virion. The earlier vaccine studies using the

mouse model employed vaccines with intact virus particles; and (2) to obtain information in this animal model on the influenza strains which are currently circulating including the A/Port Chalmers/1/73 (H_3N_2) and B/Hong Kong/5/72 strains.

Materials and methods

Vaccines

Commercially produced licensed vaccines were used in this study. The vaccines were prepared so that they contained a total of at least 1200 chick cell agglutinating units (CCA) of viral antigen per 0·5 ml when compared to a standard vaccine reference as described by Tauraso, O'Brien and Seligmann (1969). The vaccines contained at least 700 CCA of the A/Port Chalmers/1/73 antigen and 500 CCA of B/Hong Kong/5/72 antigen per 0·5 ml. Three vaccines were produced as whole virus vaccines, concentrated and purified by rate zonal centrifugation (Reimer *et al.*, 1967). One vaccine was a whole virus vaccine prepared by column chromatography and two vaccines were split-product preparations, one having been treated with Tween-80 and ether (Cromwell *et al.*, 1969), the other with tri-N-butyl phosphate (Neurath *et al.*, 1970).

Mice

Inbred 3–4-week-old male CFW strain mice purchased from Carworth Farms were inoculated i.p. with 0·5 ml of vaccine diluted in phosphate buffered saline, pH 7·2 (PBS). Groups of fifteen mice each were given 10^{-1}, 10^{-2} and 10^{-3} dilutions of the six vaccines tested.

Mouse-adapted influenza viruses

Prototype strains of egg-grown influenza viruses (A/Port Chalmers/1/73 and B/Hong Kong/5/72) were administered intranasally as undiluted allantoic fluid to ten 3-week-old CFW mice. Forty-eight to 72 hr later, three mice were killed; their lungs were homogenized and a 10% suspension was then used to pass the virus to another ten mice. Virus, 0·1 ml, was administered intranasally under ether anaesthesia. The seven remaining mice were observed for survival. This process was repeated serially. When it was observed during this serial passage that a majority of mice died in 1 week with generalized pulmonary consolidation, the lung suspension employed for that mouse passage was inoculated into the allantoic cavity of embryonated eggs to prepare a pool of virus virulent for mice. The harvested fluids were pooled, clarified and used for challenge experiments involving mice. The mouse-adapted virus was characterized by haemagglutination-inhibition (HI) testing with monospecific antisera prepared in chickens, and sera from mice infected with the stock virus were characterized with specific viral haemagglutinating antigens. These stock mouse-adapted virus pools were stored at −70°C until use. The egg infectious dose titre (EID_{50}) of the mouse-adapted A/Port Chalmers was 6·5 $\log_{10}$/0·1 ml and the B/Hong Kong strain titre 5 $\log_{10}$/0·1 ml.

Challenge studies

Four weeks after administration of vaccine or vaccine diluent, approximately 30 50% mouse lethal doses (LD_{50}) of virus diluted in PBS were administered intranasally under either anaesthesia with care to ensure inhalation of the drop. Mice were 3–4 weeks old at the time of immunization, and were therefore 7–8 weeks old when challenged. Most deaths occurred between days 4 and 7 after challenge, with a range of 2–8 days. Observations were continued for 14 days after challenge. Differences in survival between groups were analysed by the chi-square test. Animals that died from anaesthesia or bleeding were excluded from analysis. Antibody assays were performed in microtitre plates by HI using 4 units of specific viral antigen, sera treated with heat (56°C, 30 min), receptor destroying enzyme and chick red blood cells. Individual sera were titrated for antibody titres. Statistical significance of differences in antibody titres was determined by Student's *t*-test (Snedecor and Cochran, 1967).

Results

The vaccines used in these experiments were coded so that the individuals measuring the serological response and challenge results would not know the identity of the vaccines being tested. Table 1 outlines the vaccines used, their potency as measured in the CCA test, and the type of production process used to make the vaccines. For the sake of consistency and clarity the vaccines are listed as they have been described earlier (Barry, Staton and Mayner, 1974).

Data presented in Fig. 1 summarize the results of an experiment in which mice were given serial dilutions of each of the vaccines described in Table 1 and were challenged with approximately 30 LD_{50} of A/Port Chalmers/1/73 mouse-adapted virus 4 weeks after immunization. On the day before challenge, the mice were bled retro-orbitally and the individual sera were used to assay HI antibody. Twenty-six of thirty control mice which had been injected with diluent, died from the mouse virulent virus. All four of the whole virus vaccines induced significant resistance to this challenge dose of mouse-adapted virus at the 10^{-1} dilution of vaccine which contained approximately 70 CCA units of the homotypic A/Port Chalmers antigen, but the two split-product vaccines (ET, TBP) did not protect at this dilution. Two of the whole virus vaccines conferred protection against challenge at the higher dilutions

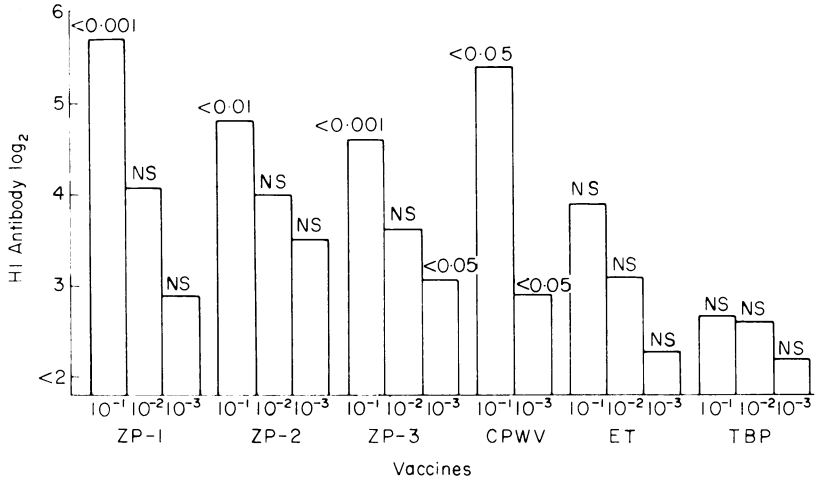

FIG. 1. Antibody titre and protection against challenge in mice given influenza vaccine (A/Port Chalmers). Geometric mean haemagglutination inhibition antibodies 28 days after immunization with indicated dilution of vaccines.

The figures shown above the mean antibody titre graphs represent the statistical significance of protection against challenge with mouse-adapted virus day 29 after immunization.

TABLE 1. Description of vaccines *

Vaccine production	CCA units $\frac{V}{R}$ ratio†
Zonally purified whole virion (ZP-1)	1·0
Zonally purified whole virion (ZP-2)	1·1
Zonally purified whole virion (ZP-3)	1·2
Chromatographed purified whole virion (CPWV)	1·0
Ether treated split-product (ET)	1·4
Tri-N-butyl phosphate treated split-product (TBP)	1·3

* Prepared to contain at least 700 CCA of A/Port Chalmers/1/73, and 500 CCA of B/Hong Kong/5/72 per 0·5 ml (dose for adult humans).

† $\frac{V}{R}$ = $\frac{\text{CCA determination of vaccine}}{\text{CCA determination of the reference (mean reference titre was 2400 CCA/1·0 ml).}}$

tested. These results compare protection of vaccinated mice against lethal challenge to control unvaccinated mice.

When one compares the protection induced by the four whole virus vaccines to that induced by the two split-product vaccines, the whole virus vaccines afforded significantly greater protection. At the 10^{-1} vaccine dilution, 70% of the recipients of whole virus vaccines survived this challenge but only 14% of the recipients of split-product vaccines survived ($\chi^2 = 21{\cdot}3$, $P < 0{\cdot}001$). At the higher vaccine dilutions the protection induced by whole virus vaccines was reduced but still significantly better (28% *v.* 10% survival, $\chi^2 = 4{\cdot}57$, $P < 0{\cdot}05$) than was observed in recipients of split-product vaccines.

Figure 1 also presents the antibody titres of mice in the various groups at the time of challenge. Six vaccines were tested at three dilutions, thus there were eighteen groups of vaccinated animals that were challenged. One group of animals (CPWV at 10^{-2} dilution) was not included in the experiment owing to an error. Only two of the thirteen groups of vaccinated animals which had mean antibody titres of $\leqslant 1:17$ ($\leqslant 4{\cdot}1$ $\log_2$) were significantly protected ($P < 0{\cdot}05$); however, the four groups which had antibody titres of $\geqslant 1:24$ ($\geqslant 4{\cdot}6$ $\log_2$) were significantly protected. Thus, the induction of this level of HI antibodies to A/Port Chalmers was associated with significant protection against this lethal challenge dose.

A similar experiment was then performed using the same bivalent vaccines and the mouse-adapted B/Hong Kong virus challenge. The mean antibody titres from each vaccine dilution are shown in Fig. 2. Again the animals were bled the day before challenge, 28 days post-immunization, and HI antibody assay was performed on the individual sera. Figure 2 also demonstrates the protection against a challenge dose of approximately 30 LD_{50} of B/Hong Kong/5/72 mouse-adapted virus. The three dilutions of vaccine contained at least 50, 5 or 0·5 CCA units per dose. The six vaccines induced antibody and significant protection against challenge at the highest dose tested when compared to the control groups of mice, in which 24 of 27 mice died after receiving this

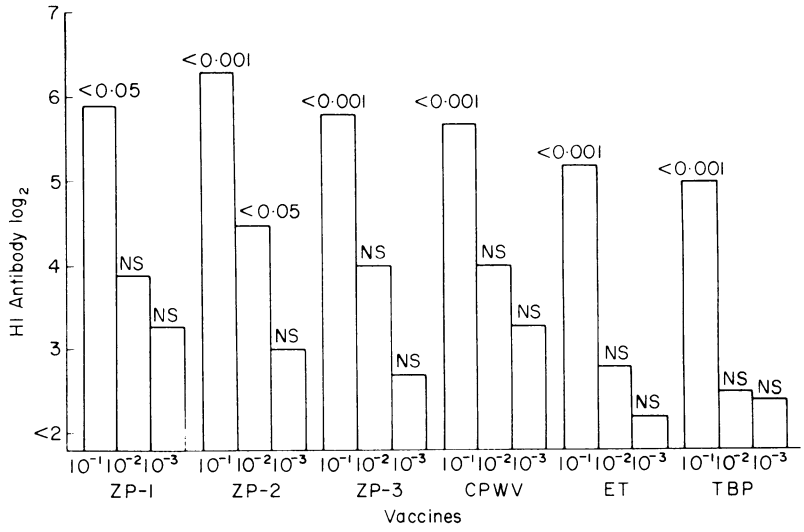

FIG. 2. Antibody titre and protection against challenge in mice given influenza vaccine (B/Hong Kong). Geometric mean haemagglutination inhibition antibodies 28 days after immunization with indicated dilution of vaccines. The figures shown above the mean antibody titre graphs represent the statistical significance of protection against challenge with mouse-adapted virus day 29 after immunization.

TABLE 2. Geometric mean antibody titres to A/Port Chalmers antigen induced by influenza vaccines

	Mean *	s.d.	Mean	s.d.	*P*†
Vaccine dilution	ET		ZP-1,2,3; CPWV; TBP		
10^{-1}	3·9	0·99	4·6	1·25	<0·05
10^{-2}	3·1	0·59	3·6	0·93	NS
10^{-3}	2·3	0·49	2·9	0·74	<0·01
Vaccine	TBP		ZP-1,2,3; CPWV; ET		
10^{-1}	2·7	0·46	4·9	1·00	<0·001
10^{-2}	2·6	0·63	3·7	0·82	<0·001
10^{-3}	2·2	0·41	3·0	0·72	<0·001
Vaccine	ET; TBP		ZP-1,2,3; CPWV		
10^{-1}	3·3	0·95	5·1	0·83	<0·001
10^{-2}	2·8	0·65	3·9	0·78	<0·001
10^{-3}	2·3	0·45	3·1	0·69	<0·001

* Geometric mean and standard deviation from the mean, HI antibody titre ($\log_2$).
† Statistical significance (Student's *t*-test) between geometric mean HI antibody titres.

challenge dose. At the 10^{-2} and 10^{-3} dilutions of the vaccines, significant protection was observed in only one group of mice, ZP-2 at 10^{-2} dilution. In this challenge experiment all seven groups of mice with a geometric mean antibody titre of $\geqslant 4{\cdot}5$ $\log_2$ were significantly protected against challenge; however, none of the ten groups of animals which had antibody titres of $\leqslant 4{\cdot}0$ $\log_2$ was protected against this challenge.

The relationship between the induction of levels of serum antibody titre of 1 : 24 and significant protection against these lethal challenge doses was highly significant for both the A/Port Chalmers/1/73 and B/Hong Kong/5/72 experiments ($P < 0{\cdot}001$). This relationship between the levels of serum HI antibody induced and protection against challenge applies to all of the vaccines tested, including the split-product vaccines. However, the geometric mean antibody titres observed in mice given the split-product vaccines were significantly lower than that induced by the four whole virus vaccines.

In Table 2 data are presented concerning the mean A/Port Chalmers/1/73 antibody titres 28 days after vaccination of the mice given split-product vaccines ET and TBP and compares them to the antibody titres measured in mice inoculated with the whole virus vaccines. It can be seen that both split-product vaccines were significantly less immunogenic in mice

TABLE 3. Geometric mean antibody titres to B/Hong Kong antigen induced by influenza vaccines

	Mean *	s.d.	Mean	s.d.	P †
Vaccine dilution	ET		ZP-1,2,3; CPWV; TBP		
10^{-1}	5·2	1·3	5·8	1·1	NS
10^{-2}	2·8	0·83	3·8	1·2	<0·01
10^{-3}	2·2	0·41	3·0	0·92	<0·01
Vaccine dilution	TBP		ZP-1,2,3; CPWV; ET		
10^{-1}	5·0	1·5	5·8	0·98	<0·05
10^{-2}	2·5	0·64	3·9	1·1	<0·001
10^{-3}	2·4	0·65	2·9	0·93	NS
Vaccine dilution	ET; TBP		ZP-1,2,3; CPWV		
10^{-1}	5·1	1·4	5·9	0·86	<0·01
10^{-2}	2·7	0·73	4·1	1·1	<0·001
10^{-3}	2·3	0·54	3·1	0·93	<0·001

* Geometric mean and standard deviation from the mean, HI antibody titre ($\log_2$).
† Statistical significance (Student's *t*-test) between geometric mean HI antibody titres.

TABLE 4. Percentage survival from challenge with A/Port Chalmers virus following vaccination*

			Challenge dose (LD_{50})		
			100	10	1
Dilution	Vaccine	Antibody †	% Survival (*P*)	% Survival (*P*)	% Survival (*P*)
10^{-1}	ZP-2	4·5	100 (<0·01)	100 (<0·001)	100 (<0·05)
	TBP	2·7 ($P<0·001$)	60 (NS)	80 (<0·001)	100 (<0·05)
10^{-2}	ZP-2	3·4	80 (<0·05)	100 (<0·001)	90 (NS)
	TBP	2·5 ($P<0·001$)	70 (NS)	77 (<0·01)	100 (<0·05)
10^{-3}	ZP-2	2·5	62 (NS)	89 (<0·001)	100 (<0·05)
	TBP	2·13 ($P<0·025$)	30 (NS)	30 (NS)	90 (NS)

* In this experiment ten animals were used per vaccine dilution. Deaths due to pre-challenge bleeding or ether occurred in some groups, and these deaths were not attributed to virus challenge.
† $\log_2$ geometric mean antibody titre of serum on day before challenge.

than the other vaccines, confirming earlier work from this laboratory (Barry *et al.*, 1974). This is evident when a comparison is made between the individual split-product vaccines and the other five vaccines, including the four whole virus and the other split-product vaccine. None of the whole virus vaccines was significantly less immunogenic than the other five vaccines when they were compared in this fashion.

In Table 3, the mean antibody titres induced by the split-product vaccines to B/Hong Kong antigen are shown and compared with the antibody response induced by the whole virus vaccines. In most dilutions vaccines ET and TBP produced significantly lower levels of HI antibody than did the other vaccines, and in every dilution they produced lower levels of antibody. Combining the antibody responses in mice given the two split-product vaccines and comparing these results to the antibodies induced by the four whole virus vaccines increased the level of statistical significance of these differences. As noted above in the experiment with A/Port Chalmers virus, none of the whole virus vaccines induced significantly lower antibodies than did the other five vaccines given at any dilution.

The above protection experiments utilized a single challenge dose of virus which killed over 90% of the control mice. We then performed a study to determine the effect of the dose of challenge virus on protection. A/Port Chalmers/1/73 challenge virus was administered to mice which had been previously immunized with 10^{-1}, 10^{-2} and 10^{-3} dilutions of two of the same bivalent vaccines used in the above studies, one a whole virus vaccine (ZP-2) and the other a split-product vaccine (TBP). A summary of the protection results is presented in Table 4. At the lower challenge doses containing approximately 1 LD_{50}, both vaccines protected; however, with a challenge dose of 10 LD_{50} the split-product vaccine did not protect at the 10^{-3} dilution. At the highest challenge dose, only the 10^{-1} and 10^{-2} doses of the whole virus vaccine conferred significant protection.

The antibody response in these animals is also shown in Table 4; as was demonstrated in the earlier experiments, the whole virus vaccine induced a higher antibody response, and this antibody response was associated with protection against high challenge doses. The split-product vaccine induced a lower antibody response and failed to protect against the highest challenge dose, but it did provide some protection at a lower challenge dose of virus.

Discussion

These results confirm and extend earlier observations made on the immunizing and protective effect of influenza vaccines. The decreased antibody response in mice given split-product vaccines when compared to whole virus vaccines, despite their possessing similar haemagglutinin titres as measured by quantitative CCA testing with a reference, may be due to the fact that mice are immunological virgins for influenza virus. Adequate immunological response on primary exposure to antigen may require the presence of either whole virion, as observed in this study, or the addition of adjuvants as others have reported (Davenport, 1968).

There have been no reports comparing protection conferred against lethal challenge with these modern influenza vaccines. However, the present study confirms earlier reports which provided evidence that formalin-inactivated whole virus influenza vaccines conferred specific protection against subsequent challenge. Hinz *et al.* (1972) observed a lower HI antibody response to a Tween-ether-disrupted A/Aichi/68 vaccine than to vaccine made with intact virus. These workers also found the ether-disrupted vaccine to be less protective than the whole virus vaccine in mouse challenge experiments with the A/Aichi/68 strain. The present data confirm that observation and demonstrate that another type of split-product vaccine is also less immunogenic and less protective than whole virus vaccines in this model.

The correlation between the amount of specific serum haemagglutinating antibodies and protection against subsequent challenge seen in this experimental model system has also been observed in man (Hobson *et al.*, 1972). There are problems associated with extrapolating from laboratory animal data to man, but the fact that the amount of serum antibody present in mice and man is a reliable indication of protection against influenza infection should be noted. It appears that mice, without prior natural immunological experience with influenza viruses, represent a reasonable laboratory model for immunologically virgin man. Such a circumstance is present when a major shift in both surface antigens occurs, or in young children on initial influenza virus infection. It has not been possible to compare the immunogenic effect of modern highly purified whole and split-product vaccines in immunologically virgin adults because influenza virus N_2 neuraminidase antigen has not changed completely since 1957, and the H_3 haemagglutinin subtypes have been circulating since 1968; therefore, older children and adults have a broad background of natural exposure to these antigens. Thus, immunogenicity studies performed in individuals give evidence for the efficacy of the vaccines being tested against this background of experience but cannot be taken as evidence for vaccine effectiveness in animals or man without prior natural exposure to the same or related strains. Limited studies have been performed in young children with intact or disrupted inactivated influenza vaccines. Some of these studies were performed with older experimental H_2N_2 vaccines and indicated that split-product vaccines were associated with fewer systemic reactions, but they and their whole virus counterparts were poor immunogens. More recent studies performed with zonally purified whole virus vaccines have indicated adequate immunogenicity, but reported higher rates of clinical reactivity (Wright *et al.*, 1975; Marine and Stuart-Harris, 1975). These difficulties encountered in determining the efficacy of influenza vaccines in non-immune man, along with some of the similarities observed between the degree of protection associated with the antibody levels observed in mice and man, lead to a tentative conclusion that certain split-product vaccines, without adjuvant, may be less immunogenic for non-immune individuals. More information is needed on the comparative protective efficacy of split-product and whole virus vaccines in immunologically inexperienced subjects.

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