J. Hyg., Camb. (1975), **75**, 341
Printed in Great Britain

A new, surface-antigen-adsorbed influenza virus vaccine

I. Studies on immunogenicity in hamsters

By R. JENNINGS, C. W. POTTER and C. McLAREN
Academic Division of Pathology (Virology), University of Sheffield Medical School, Beech Hill Road, Sheffield S10 2RX
and MARGARET BRADY
Evans Biologicals Ltd, Speke, Liverpool L24 9JD

(*Received* 8 *April* 1975)

SUMMARY

The ability of a new, surface-antigen-adsorbed influenza virus vaccine to induce serum antibody in hamsters, and to protect these hamsters against subsequent homologous virus challenge, is reported. In addition, similar studies in hamsters have also been carried out using the surface antigen material prior to adsorption to the aluminium hydroxide carrier. The new, adsorbed vaccine is at least as effective as inactivated saline influenza virus vaccine in inducing serum antibody and protection in hamsters; the unadsorbed surface antigen material, however, did not confer protection to hamsters challenged subsequently with homologous virus.

INTRODUCTION

Previous reports from this laboratory have described the use of the hamster as an experimental model for examining the antibody response to inactivated influenza vaccines (Jennings & Potter, 1973; Potter, Jennings, Marine & McLaren, 1973). Earlier workers (Taylor & Parodi, 1942; Friedewald & Hook, 1948) have found that influenza viruses replicate in the lungs of hamsters and can be recovered from this tissue in high titre. These findings suggest that hamsters could provide a useful model system for the study of influenza infection and immunization.

In the present study we have examined the serum antibody response of hamsters to an inactivated whole influenza virus vaccine; to virus subunit material containing haemagglutinin and neuraminidase antigens and to subunit material adsorbed to an alhydrogel carrier. These vaccines represent a series of materials in the production of an adsorbed, subunit vaccine. It is hoped that the final material would be an improved vaccine, since purified, subunit vaccines are less toxic than whole virus vaccines (Webster & Laver, 1966; Brandon, Barrett, Hook & Lease, 1967), and a carrier, by aggregating the relatively small surface antigens of the influenza virus, would increase both the antigenicity and the duration of the antigenic stimulus of the virus subunits. All the immunized hamsters were challenged with live virus to determine the immunizing effects of the various vaccines.

https://doi.org/10.1017/S0022172400024402 Published online by Cambridge University Press

MATERIALS AND METHODS

Viruses

Influenza virus A/England/42/72 (H3N2) was obtained from Dr G. C. Schild, National Institute of Medical Research, Mill Hill, London. This virus was used to inoculate hamsters intranasally; 3 days after inoculation, virus was recovered from lung suspensions, and 0·2 ml. of a 40 % lung suspension was used to inoculate further hamsters. The virus was passaged eleven times in this manner. After the final passage, lung suspensions were inoculated into 10-day embryonated hen's eggs. The eggs were incubated for 3 days at 35° C. and the infected allantoic fluids collected, pooled and stored at −80° C. This pool, which contained $10^{9\cdot2}$ EID 50/ml. of infective virus, was identified as A/England/42/72 by haemagglutination inhibition (HI) using monospecific ferret antisera.

Influenza virus A/FM/1/74 (H1N1) was a strain of virus maintained in our laboratory by allantoic inoculation of 10-day embryonated eggs. The virus pool used in the present study contained $10^{8\cdot9}$ EID 50/ml.

Vaccines

All the vaccine materials were supplied by Dr I. Furminger, Evans Biologicals Ltd, Speke, Liverpool. Inactivated, whole influenza virus vaccine containing 16,000 international units (i.u.) per ml. was prepared by zonal centrifugation of allantoic fluids from infected embryonated eggs. Haemagglutinin (HA) and neuraminadase (N) surface antigens, free of virus-core material were prepared by Triton treatment of purified whole virus vaccine; this material contained 1300 i.u./ml. A surface-antigen-adsorbed vaccine containing 600 i.u./ml. was prepared by adsorption of the purified HA and N antigens onto alhydrogel (aluminium hydroxide, $Al(OH)_3$).

Experimental design

Studies were performed using 2- to 3-month-old Syrian hamsters obtained from a single, randomly bred colony at the University of Sheffield, or from accredited dealers. A blood specimen was collected from the orbital sinus of each hamster before use. Some hamsters were then infected intranasally with 0·2 ml. of influenza virus A/FM/1/47 containing $10^{4\cdot5}$–$10^{6\cdot5}$ EID 50, since previous studies have shown that hamsters respond better to inactivated influenza vaccine after prior virus infection with heterotypic influenza virus (Jennings & Potter, 1973). Groups of 4–6 normal or pre-infected hamsters were immunized intramuscularly with dilutions of one of the vaccines or the subunit material in an 0·5 ml. volume, and 3 weeks later a further blood sample was taken from each animal. After this, all the hamsters were inoculated intranasally with 0·2 ml. of A/England/42/72 virus. The dose of A/England/42/72 virus used for this challenge infection was 100 HID (hamster infective doses) per 0·2 ml.; this was established by prior titration in normal hamsters. The lungs were collected from the animals 3 days after infection, pooled, and a 40% (v/v) suspension titrated for virus content.

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Virus isolation

Virus was recovered from hamster lung suspensions or nasal washings after infection. Lung suspensions were prepared from pooled lungs taken from hamsters killed by cervical dislocation. The lungs were washed in PBS, ground with a pestle and mortar in carborundum powder and a 40% (v/v) suspension prepared. This was clarified by centrifugation at 1500 rev./min. Hamster nasal washings were obtained by the dropwise instillation of 1·0 ml. of PBS containing 1% bovine serum albumin (BSA) and antibiotics into hamster nostrils using a syringe. The animals were held firmly by the skin at the back of the neck so that the jaws remained open. The diluent flowed through the nasal passages into the mouth and was collected from the throat using a Pasteur pipette. Suspensions and washings were stored at −80° C.

The titre of virus in nasal washings and lung suspensions was estimated using the allantois-on-shell (AOS) method (Fazekas de St Groth, Withell & Lafferty, 1958).

Titrations for serum antibody

Haemagglutination-inhibition (HI) tests were carried out using methods described previously (Jennings & Potter, 1973). Anti-neuraminidase antibody assays were carried out using standard World Health Organization methods, with standard WHO reagents (Aymard-Henry *et al.* 1973).

RESULTS

Titration of A/England/42/72 virus in hamsters

Dilutions of influenza A/England/42/72 virus which had been serially passed in hamsters were inoculated intranasally into groups of normal hamsters. Three days after infection, nasal washings and lungs were collected from some hamsters in each group, and titrated for virus. Sera from the remaining hamsters, together with sera obtained before infection, were then tested for HI antibody response. The results are shown in Table 1. Virus was isolated from nasal washings and lung suspensions of hamsters infected with all the virus dilutions to 30 EID 50. A serum HI antibody response to A/England/42/72 virus was also observed in all hamsters infected with 30 or more EID 50, but not for hamsters inoculated with a smaller dose of virus. Thus, virus isolation and serological response show complete correlation.

The temperature of the hamsters was measured, per rectum, for 3 successive days before and 3 successive days after virus infection, but no significant change was observed in any individual animal. Thus, hamsters did not show a febrile response to influenza infection.

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Table 1. *Titration of A/England/42/72 influenza virus in hamsters*

Virus Inoculum (log_{10}EID 50/ml.)	Hamster no.	Titre* of virus isolated from: Nasal washings (3 days post-infection)	Titre* of virus isolated from: Lung suspensions (3 days post-infection)	Serum HI antibody response at 21 days post-infection
4·5	1	5·80	4·19	ND
	2	4·57	5·37	ND
	3	ND	ND	<10–30
	4	ND	ND	<10–30
	5	ND	ND	<10–15
3·5	1	>5·80	3·46	ND
	2	>5·80	5·46	ND
	3	ND	ND	<10–30
	4	ND	ND	<10–30
	5	ND	ND	<10–60
2·5	1	3·80	>5·80	ND
	2	4·55	4·80	ND
	3	ND	ND	<10–30
	4	4·57	ND	<10–60
	5	ND	ND	<10–240
1·5	1	3·01	2·30	ND
	2	3·80	2·30	ND
	3	3·50	2·30	ND
	4	ND	ND	<10–15
	5	ND	ND	<10–60
	6	ND	ND	<10–60
0·5	1	<0·50	<0·50	ND
	2	ND	<0·50	ND
	3	ND	ND	—
	4	<0·50	ND	—

ND, Not done. * Log_{10}/ml.

Comparison of titration of A/England/42/72 whole virus and adsorbed vaccines in hamsters

A/England/42/72 whole virus vaccine

Groups of normal hamsters, and hamsters infected 3 weeks earlier with live, A/FM/1/47 influenza virus, were test bled and inoculated intramuscularly with varying doses of A/England/42/72 whole virus vaccine. Serum specimens were taken 3 weeks later and tested for HI and NI antibodies to A/England/42/72 virus and for HI antibodies to A/FM/1/47. Haemagglutination inhibiting antibodies to A/FM/1/47 virus, ranging in titre from 1/30 to 1/240, were present at the time of immunization in all hamsters pre-infected with this virus, but the antibody titres did not change significantly after immunization with A/England/42/72 vaccine.

The serum antibody responses to immunization with A/England/42/72 whole virus vaccine are shown in Table 2. Only two of four normal hamsters immunized with 600 i.u., and one of four immunized with 60 i.u. of vaccine produced demonstrable serum HI antibody; none developed demonstrable NI antibody. In contrast, all hamsters pre-infected with A/FM/1/47 virus produced serum HI antibody

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https://doi.org/10.1017/S0022172400024402 Published online by Cambridge University Press

[Table 2. *Response of hamsters to immunization with influenza A/England/42/72 whole virus vaccine*

Vaccine Dose (i.u.)	Hamster no.	Normal hamsters: HI antibody response to A/Eng/42/72	Normal hamsters: NI antibody response to A/Eng/42/72	Normal hamsters: Virus recovery after challenge (log_{10}/ml.*)	Pre-infected hamsters: HI antibody response to A/Eng/42/72	Pre-infected hamsters: NI antibody response to A/Eng/42/72	Pre-infected hamsters: Virus recovery after challenge (log_{10}/ml.)
600	1	<10–30	—	—	<10–160	<10–60	
	2	<10–15	—	—	<10– 80	—	<0·50
	3	—†	—	<0·50	<10–160	—	
	4	—	—	—	<10– 80	—	
60	1	<10–15	—	—	<10–240	<10–60	
	2	—	—	—	<10– 30	—	<0·50
	3	—	—	<0·50	<10– 15	—	
	4	—	—	—	<10– 30	—	
6	1	—	—	—	<10– 15	—	
	2	—	—	—	—	—	<0·50
	3	—	—	2·15	—	—	
	4	—	—	—	—	—	
0·6	1	—	—	—	—	—	
	2	—	—	—	—	—	3·37
	3	—	—	3·43	—	—	
	4	—	ND	—	—	ND	

ND, Not done.

* Virus estimated by allantois-on-shell titrations of pooled, 40 % hamster lung suspensions.

† <10 to <10.

in response to immunization with 600 or 60 i.u. and one of four produced antibody in response to 6·0 i.u. of A/England/42/72 virus vaccine. The serum HI antibody titres were also higher in pre-infected hamsters, and two of these hamsters showed demonstrable NI antibody after immunization (Table 2).

Three weeks after immunization the hamsters were inoculated intranasally with live, homologous A/England/42/72 virus; lung suspensions were collected 3 days later and titrated for virus. The results are shown in Table 2. Hamsters pre-infected with heterotypic A/FM/1/47 influenza virus and then immunized with A/England/42/72 whole virus vaccine were more resistant to challenge infection with homologous virus than hamsters that had been given vaccine only. This result was not due to the initial infection, since pre-infection with A/FM/1/47 virus alone gave no immunity to challenge infection. Virus could not be recovered from lung suspensions of pre-infected hamsters immunized with 600, 60 or 6·0 i.u. of A/England/42/72 vaccine, but could be recovered from hamsters immunized with 0·6 i.u. (Table 2). In contrast, virus was recovered from normal hamsters immunized with either 6·0 or 0·6 i.u. of vaccine.

Adsorbed A/England/42/72 surface antigen vaccine

The titration of surface antigen A/England/42/72 vaccine adsorbed to alhydrogel carrier was carried out as described for whole virus vaccine. All A/FM/1/47-infected hamsters showed homologous serum HI titres of 1/60 to 1/240 before immunization, and these titres remained unchanged after immunization. The results of immunization and challenge infection of hamsters is shown in Table 3. All normal hamsters inoculated with 600 i.u. of vaccine and two of four animals inoculated with 60 i.u. of vaccine developed demonstrable serum HI antibody; all hamsters given 600 or 60 i.u. of vaccine and three of four animals given 6 i.u. of vaccine developed NI antibody. Thus, this vaccine was more antigenic in hamsters than whole virus vaccine (Table 2). Pre-infection with A/FM/1/47 virus did not enhance the serum HI antibody response to the adsorbed A/England/42/72 surface antigen vaccine; indeed, this vaccine appeared more effective in inducing antibody in normal than in pre-infected hamsters. Thus, only one of four hamsters pre-infected with A/FM/1/47 virus developed NI antibody in response to immunization with 6 i.u. of vaccine, whilst three of four normal hamsters produced NI antibody.

In contrast to the serum antibody response to immunization, pre-infected hamsters were more resistant to subsequent challenge with influenza virus A/England/42/72 (Table 3). Virus was not recovered from the lungs of pre-infected hamsters immunized with 600, 60 or 6 i.u. of A/England/42/72 vaccine; in contrast, virus was recovered from normal hamsters inoculated with 6 i.u. of vaccine.

Comparison of titration of A/England/42/72 whole virus vaccine and the surface antigens alone, in hamsters

A/England/42/72 whole virus vaccine

The titrations of A/England/42/72 whole virus vaccine was repeated as a parallel control for the study of the subunit material, and to test the reproducibility of this system; the same experimental design was used as described earlier. All

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Table 3. *Response of hamsters to immunization with A/England/42/72 subunit material adsorbed to alhydrogel carrier*

Vaccine Dose (i.u.)	Hamster no.	Normal hamsters: HI antibody response to A/Eng/42/72	Normal hamsters: NI antibody response to A/Eng/42/72	Normal hamsters: Virus recovery after challenge (log_{10}/ml.*)	Pre-infected hamsters: HI antibody response to A/Eng/42/72	Pre-infected hamsters: NI antibody response to A/Eng/42/72	Pre-infected hamsters: Virus recovery after challenge (log_{10}/ml.)
600	1	<10–30	<10–160	<0·50	<10–120	<10–160	<0·50
	2	<10–30	<10–240		<10–120	<10–240	
	3	<10–15	<10–240		—	<10–160	
	4	<10–20	<10–160		—	<10–160	
60	1	<10–30	<10–240	<0·50	<10–120	<10– 60	<0·50
	2	<10–15	<10– 20		—	<10– 80	
	3	—†	<10–240		—	—	
	4	—	ND		—	ND	
6	1	—	<10–160	4·02	<10– 60	ND	<0·50
	2	—	<10–240		—	—	
	3	—	<10– 20		—	—	
	4	—	—		—	—	
0·6	1	—	—	4·80	—	<10– 20	3·37
	2	—	—		—	—	
	3	—	—		—	—	
	4	—	—		—	—	

ND, Not done.

* Virus estimated by allantois-on-shell titrations of pooled, 40 % hamster lung suspensions.

† <10 to <10.

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Table 4. *Response of hamsters to immunization with influenza A/England/42/72 whole virus vaccine*

Vaccine Dose (i.u.)	Hamster no.	Normal hamsters: HI antibody response to A/Eng/42/72	Normal hamsters: NI antibody response to A/Eng/42/72	Normal hamsters: Virus recovery after challenge (log_{10}/ml.*)	Pre-infected hamsters: HI antibody response to A/Eng/42/72	Pre-infected hamsters: NI antibody response to A/Eng/42/72	Pre-infected hamsters: Virus recovery after challenge (log_{10}/ml.)
600	1	<10–60	—		<10– 20	<10–160	
	2	<10–20	—		<10– 40	—	
	3	—†	—		<10– 60	ND	
	4	<10–60	—	<0·50	<10–240	<10–160	<0·50
	5	<10–30	—		<10– 30	<10–160	
	6	<10–15	—		<10– 30	<10–160	
60	1	<10–30	—		<10– 10	<10–160	
	2	—	<10–20		<10– 30	—	
	3	—	—		—	—	
	4	<10–15	—	<0·50	<10–120	<10– 20	<0·50
	5	—	—		<10– 30	<10–160	
	6	—	—		<10– 30	—	
6	1	—	—		<10–120	<10– 30	
	2	—	—		—	<10– 20	
	3	—	—		—	—	
	4	—	—	2·15	<10–120	<10– 20	<0·50
	5	—	—		—	—	
	6	—	—		—	—	
0·6	1	—	—		—	—	
	2	—	—		—	—	
	3	—	—		—	—	
	4	—	—	<0·50	—	—	4·30
	5	—	—		—	—	
	6	—	—		—	—	

ND, Not done.

* Virus estimated by allantois-on-shell titrations of pooled, 40 % hamster lung suspensions.

† <10 to <10.

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Table 5. *Response of hamsters to immunization with influenza A/England/42/72 subunit material*

Vaccine Dose (i.u.)	Hamster no.	Normal hamsters: HI antibody response to A/Eng/42/72	Normal hamsters: NI antibody response to A/Eng/42/72	Normal hamsters: Virus recovery after challenge (log_{10}/ml.*)	Pre-infected hamsters: HI antibody response to A/Eng/42/72	Pre-infected hamsters: NI antibody response to A/Eng/42/72	Pre-infected hamsters: Virus recovery after challenge (log_{10}/ml.)
600	1	<10–15	<10–120	2·96	—	—	4·57
	2	<10–20	<10– 40		—	—	
	3	<10–20	—		—	—	
	4	<10–15	—		<10–120	<10–20	
	5	ND	ND		—	—	
	6	ND	ND		—	—	
60	1	<10–10	—	3·06	<10– 30	—	5·80
	2	<10–10	—		—	—	
	3	<10–40	—		—	—	
	4	—†	—		<10– 60	—	
	5	<10–15	—		—	—	
	6	—	—		—	—	
6	1	—	—	4·80	<10– 20	—	4·46
	2	—	—		—	—	
	3	—	—		—	—	
	4	—	—		—	—	
	5	—	—		—	—	
	6	—	—		—	—	
0·6	1	—	—	4·46	—	—	5·10
	2	—	—		—	—	
	3	—	—		—	—	
	4	—	—		—	—	
	5	—	—		—	—	
	6	—	—		—	—	

ND, Not done.

* Virus estimated by allantois-on-shell titrations of pooled, 40 % hamster lung suspensions.

† <10 to <10.

pre-infected hamsters had serum HI antibody to A/FM/1/47 virus at the time of immunization, and these titres remained unchanged throughout. The results are shown in Table 4, and were very similar to those of Table 2. Thus, five of six normal hamsters inoculated with 600 i.u. and two of six inoculated with 60 i.u. of vaccine developed detectable serum HI antibody; virus was not recovered from these hamsters after challenge infection, but virus was recovered from hamsters given smaller doses of vaccine which did not produce serum HI antibody after immunization. For hamsters pre-infected with A/FM/1/47 virus, serum antibody was found for some of the animals inoculated with 600, 60 or 6 i.u. of vaccine; virus was not recovered from these hamsters after challenge (Table 4). Thus, prior infection with A/FM/1/47 increased the antibody response to A/England/42/72 vaccine, and increased the immunity to challenge infection. This result was identical with that seen in Table 2, except for the failure to recover virus after challenge infection of normal hamsters given 0·6 i.u. of vaccine. This was clearly a technical fault, since virus was recovered from normal hamsters given 6 i.u. of vaccine (Table 4).

A/England/42/72 surface antigens

Table 5 shows the serum antibody response, and the response to A/England/42/72 challenge infection of normal and pre-infected hamsters immunized with influenza A/England/42/72 surface antigens alone. Serum HI antibody was produced in all normal hamsters inoculated with 600 i.u. and in four of six animals inoculated with 60 i.u. of vaccine. Hamsters pre-infected with A/FM/1/47 virus, all of which had serum HI antibody against A/FM/1/47 at the time of immunization, responded less well to subunit materials (Table 5). The serum NI antibody response to immunization with the subunits was poor in both normal and pre-infected hamsters.

Both pre-infected and normal hamsters given the relatively high dose of 600 i.u. of surface antigen material did not show any immunity to challenge infection with homologous A/England/42/72 virus; high titres of virus were found in pooled lung suspensions from all groups of hamsters (Table 5). This lack of protection was observed in hamsters which had demonstrable serum HI antibody to the challenge virus, at titres comparable with those found in hamsters given whole virus vaccine and which had given immunity to challenge infection.

DISCUSSION

The present paper reports the potential value of hamsters as an experimental model for the study of influenza virus vaccines. The hamster has several advantages over other animal model systems: it is less expensive, smaller and more easily handled than the ferret, and can be used in larger numbers; it is genetically more inbred and therefore results are likely to be less variable than in ferrets. The hamster may also have advantages over mice; influenza virus infection in the hamster appears more localized to the upper respiratory tract than in mice, and hamsters are highly susceptible to infection with small doses of adapted or unadapted virus. Furthermore, the antibody response and immunity of hamsters can

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be followed over long periods, and virus isolation studies made sequentially, since virus can be isolated in high titre from nasal washings. However, it is not known to what extent results obtained in hamsters are comparable to those in other animals or in man.

Hamsters were successfully infected with approximately 30 EID50 of virus given intranasally. This is a larger dose than for ferrets where infection can be achieved with approximately 3·0 EID50 of virus. Virus was recovered 3 days after infection, and serum HI and NI antibody response detected 21 days after infection.

Three A/England/42/72 vaccines were examined in hamsters; these were a zonally purified, whole virus vaccine, a Triton-split subunit material derived from the purified virus and containing only the HA and N surface antigens, and these subunits adsorbed to an alhydrogel carrier. The subunit material used in these studies was relatively pure and free from contaminating 'viral core' material as measured by polyacrylamide gel electrophoresis (J. S. Oxford, personal communication). All three vaccines induced serum antibody in hamsters. For whole virus vaccine, an enhanced antibody response was seen in hamsters pre-infected with A/FM/1/47 virus. This result was consistent with our previous studies (Jennings *et al.* 1974). Pre-infection did not cause an enhanced reaction to the split virus materials, and this may indicate that the carrier antigen on which the enhanced antibody response depended (Potter, Jennings, Rees & McLaren, 1973) was one of the discarded core proteins.

The enhanced antibody response to whole virus vaccine in hamsters pre-infected with A/FM/1/47 virus was also seen in the challenge infection. Pre-infected hamsters were immune to challenge infection after immunization with 6 i.u. of vaccine; in contrast, normal hamsters were not immune to challenge infection after immunization with this dose of vaccine. All the hamsters given the surface antigen material were successfully infected with the challenge virus, despite the presence of serum antibody at titres which, when induced with whole virus vaccine, had given immunity. This result suggests that the presence of serum antibody although a good measure of immunity, may not be the complete mechanism whereby immunity is brought about. Adsorption of the surface antigens onto the alhydrogel carrier increased their antibody-inducing capacity only slightly, but did produce immunity to challenge infection. The reasons for this effect of the carrier are not clear and indeed the mechanisms of action of alhydrogel, and other adjuvants *in vivo* are complex and poorly understood (Maillard & Bloom, 1972). However, it is known that adjuvants exert their immunological effects primarily through thymus-dependent lymphocytes (Allison & Davies, 1971; Hamaoka & Katz, 1973), and this can result in an elevated antibody response through cellular co-operation (Allison & Davies, 1971), but may also enhance other cell-mediated immune mechanisms.

It is interesting to note from these studies the protection against challenge infection afforded to hamsters by HI and NI antibodies. The results show that protection against challenge infection was only demonstrable in groups of hamsters in which one or more of the animals produced detectable amounts of serum HI antibody. Thus, of two groups of normal hamsters each inoculated with 60 i.u. of

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whole virus vaccine, only 1 of 4 and 2 of 6 animals respectively developed detectable serum HI antibody yet virus could not be recovered after subsequent challenge from these animals. This may be due to small, undetected amounts of antibody present in some animals at the time of challenge, or the presence of antibody in the lung suspensions. On the other hand, no detectable HI antibody but high NI antibody titres are found in hamsters given 6 i.u. of alhydrogel vaccine, but these animals were susceptible to challenge infection. Other studies (Schulman, Khakpour & Kilbourne, 1968; Hobson, Beare & Ward-Gardner, 1971) have reported that NI antibodies may be of less importance in protection against influenza infection in humans and animals than are HI antibodies.

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https://doi.org/10.1017/S0022172400024402 Published online by Cambridge University Press