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Relative Immunogenicity of the Cold-Adapted Influenza Virus A/Ann Arbor/6/60 (A/AA/6/60-*ca*), Recombinants of A/AA/6/60-*ca*, and Parental Strains with Similar Surface Antigens

G. A. TANNOCK,* JUDITH A. PAUL, AND R. D. BARRY

Faculty of Medicine, The University of Newcastle, New South Wales 2308, Australia

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The immunogenicity of several cold-adapted (*ca*) viruses was compared in CSL mice with that of wild-type parental viruses with similar surface antigens, according to the vaccinating dose required to clear a challenge consisting of $10^{4.5}$ 50% tissue culture infective doses of the wild-type virus. All *ca* viruses were less immunogenic than their wild-type parental strains by a factor of $10^{1.3}$ to $10^{3.4}$, probably due to the restricted capacity of *ca* viruses to replicate in the respiratory tracts of mice. However, their immunogenicity was considerably enhanced when two quite small doses were administered 3 weeks apart. The immunogenicity of *ca* viruses when administered in two doses and wild-type viruses when administered as a single dose varied according to their surface antigens. It was highest for viruses with the H2N2 A/Ann Arbor/6/60 and H3N2 A/Queensland/6/72 surface antigens and lowest for those with H1N1 A/HK/123/77 surface antigens. When two doses consisting of $10^{5.0}$ 50% tissue culture infective doses of A/Ann Arbor/6/60-*ca* were administered at an interval of 3 weeks, solid immunity was induced against the wild-type A/Ann Arbor/6/60 parental virus, two heterologous H3N2 strains, and an H1N1 strain.

Cold-adapted (*ca*) mutants of influenza A viruses have been adapted to grow in eggs or primary chicken kidney cell cultures at 25°C, a temperature restrictive for the growth of wild-type viruses (6, 7). Such a mutant of the H2N2 strain A/Ann Arbor/6/60 (A/AA/6/60-*ca*) has been proposed for use as a master strain in the development of living attenuated vaccines against influenza A (2). Recombinants of this strain and several virulent influenza A viruses have been used in a number of clinical trials and shown to be attenuated and immunogenic when administered to animals and humans (9). We recently described a sensitive clearance test for determining the immunogenicity of influenza viruses (18). The test is based on the intranasal vaccinating dose required to induce inhibition of multiplication of unadapted influenza viruses in the lungs of mice. Considerable differences in immunogenicity were demonstrated between two H3N2 influenza viruses and an H1N1 virus after each had been cloned in cell culture at 39°C. In this study, the same model was used to determine the immunogenicity of a number of recombinants of A/AA/6/60-*ca* in comparison with their parental strains. The capacity of these recombinants to induce heterologous cross-protection between influenza types and subtypes is also examined.

MATERIALS AND METHODS

Viruses. Table 1 summarizes the origin and gene composition of A/AA/6/60-*ca* and the various *ca* recombinants (determined by RNA analysis [1]) used in the present study. Wild-type viruses that were used to prepare each recombinant are also included. Each recombinant was obtained by coinfection of primary kidney cells with a wild-type virus and the cold-adapted master strain A/AA/6/60-*ca*, whose derivation from the wild-type A/AA/6/60 has been described previously (6). All *ca* recombinants, the parental A/AA/6/60-*ca*, and the wild-type parental strains were obtained from H. F. Maassab, Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor. Influenza A strains A/NT/60/68 and A/Vic/3/75 and B strain B/Singapore/222/79 were obtained from S. J. Rodda, Commonwealth Serum Laboratories, Parkville, Victoria, Australia. Except where mentioned, all viruses were grown in fertile chicken eggs for 2 days at 33 to 34°C, and the resulting stocks were put into ampoules for storage at −70°C.

Mice. Male mice have been shown in other studies to allow more rapid rates of multiplication of influenza A than female mice (M. F. Warburton, Ph. D. thesis, Australian National University, Canberra, 1963) and were used throughout. CSL white mice, randomly bred in a closed colony (16), were used, and mean weights per animal after 3, 4, 5, and 6 to 7 weeks of growth were 9, 15, 20, and 25 g, respectively. At the time of inoculation each mouse was an anesthetized by intraperitoneal inoculation with 0.2 ml of 3.33% (wt/vol) 2,2,2-tribromoethanol in 3.33% (vol/vol) tertiary amyl alcohol. Virus inocula were instilled intranasally in each mouse in a total volume of 0.05 ml, using a calibrated 0.025-ml dropping pipette. Control mice received the same volume of phosphate-buffered saline (PBS). All animals were housed at a constant temperature of 21 to 22°C in groups of not less than six. None of the viruses used in this study produced respiratory symptoms or consolidation with an increase in lung weight.

Preparation of lung and turbinate extracts. Mice were killed by increasing the anesthetizing dose 2.5 times. Lungs and/or turbinates from each mouse were removed aseptically, placed into tared bottles containing 1.0 ml of PBS with gelatin (2.5 mg ml^{-1}), penicillin (1,000 U ml^{-1}), streptomycin (1,000 μg ml^{-1}), gentamycin (50 μg ml^{-1}), and fungizone (1 μg ml^{-1}), and their weights were determined. Extracts of each organ were prepared by grinding in a chilled glass homogenizer in the presence of alumina to produce a suspension of approximately 15% (wt/vol) for lungs for 5% for turbinates. Each suspension was centrifuged at 60 × *g* for 10 min, and the supernatant was removed and stored for assay at −70°C.

Virus assays. All extract titrations were carried out in confluent 2-day cultures of the MDCK cell line prepared in

* Corresponding author.

TABLE 1. Gene composition of A/AA/6/60-*ca* and *ca* recombinants derived at 25°C[a] used in the present study

ca Virus	Surface antigen	Wild-type parent	Derivation of genes[b]							
			HA	NA	P3 (RNA1)	P1 (RNA2)	P2 (RNA3)	NP	M	NS
A/Ann Arbor (AA)/6/60-*ca*[c]	H2N2	A/AA/6/60[c]	A[d]	A	A	A	A	A	A	A
AA-CR6 clone 0	H3N2	A/Queensland (Qld)/6/72	W[d]	W	A	A	A	A	A	A
AA-CR18 clone 7	H3N2	A/Scotland (Scot)/840/74	W	W	A	A	A	A	A	W
AA-CR29 clone 2	H3N2	A/Alaska/6/77	W	W	A	A	A	A	A	A
AA-CR35 clone 2	H1N1	A/Hong Kong (HK)/123/77	W	W	A	A	A	A	A	A

[a] From Cox et al. (1).
[b] HA, Hemagglutinin; M, matrix protein; NA, neuraminidase; NS, nonstructural protein; P1 to P3, polymerases; NP, nucleoprotein.
[c] A/AA/6/60-*ca* was derived from A/AA/6/60 by cold adaptation (6).
[d] W indicates gene derived from wild-type parent; A indicates gene derived from A/AA/6/60-*ca*.

24-well Linbro tissue culture plates (Flow Laboratories, McLean, Va.). One hundred-microliter portions of each dilution were inoculated to monolayers which had been washed with PBS, and adsorption was allowed to take place at 20°C for 30 min. One milliliter of a maintenance medium consisting of a 1:1 mixture of Eagle minimal essential medium (with nonessential amino acids) and Leibovitz L-15 medium, together with glutamine (0.29 mg ml^{-1}), HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) buffer (0.01 M; pH 6.9), trypsin (Worthington Biochemical Corp., Freehold, N.J.; catalog no. 3704; 1 μg ml^{-1}), gentamycin (50 μg ml^{-1}), penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), and fungizone (1 μg ml^{-1}), was added to each culture. The plates were incubated at 34°C in a 5% CO_2 atmosphere for 5 days. Virus growth was detected by hemadsorption after the addition of 0.5% washed guinea pig erythrocytes to all cultures. Virus yields from individual organs were calculated as the 50% tissue culture infective dose ($TCID_{50}$) per tissue and are expressed throughout as the mean ± standard error of the mean (SEM) for each group of mice.

For plaque assays, duplicate MDCK monolayer cultures in six-well plates were washed with PBS and inoculated with 100-μl dilutions of each isolate. After adsorption for 45 min at 20°C, 3 ml of an overlay medium warmed at 45°C was applied. The medium consisted of Leibovitz L-15 medium with glutamine (0.15 mg ml^{-1}), HEPES buffer (0.01 M; pH 6.8), trypsin (1 μg ml^{-1}), agarose (0.9%), and antibiotics. After the overlay had hardened, each plate was incubated for 5 days in a 5% CO_2 atmosphere at 34°C. Plaques were counted without the addition of a stain.

Serology. Hemagglutinin inhibition (HI) antibody titrations of mouse sera were carried out with 4 hemagglutinating units of antigen and 0.05% chicken erythrocytes (14). Nonspecific inhibitors were removed by adsorption with 5% chicken erythrocytes overnight at 4°C, treatment with receptor-destroying enzyme overnight at 37°C, and heating at 56°C for 30 min.

RESULTS

Relative immunogenicity of recombinants of A/AA/6/60-*ca* and their wild-type parental viruses. Groups of mice were inoculated with dilutions of each *ca* recombinant or its wild-type parental virus by the intranasal route. After 4 weeks, individual mice from each group were challenged with $10^{4.5}$ $TCID_{50}$ of the homologous parental strain by the same route. All animals were sacrificed after 3 days, and extracts were prepared from individual lungs for virus assay. Optimum yields of influenza virus have been obtained in the same type of outbred mice at 3 days (19).

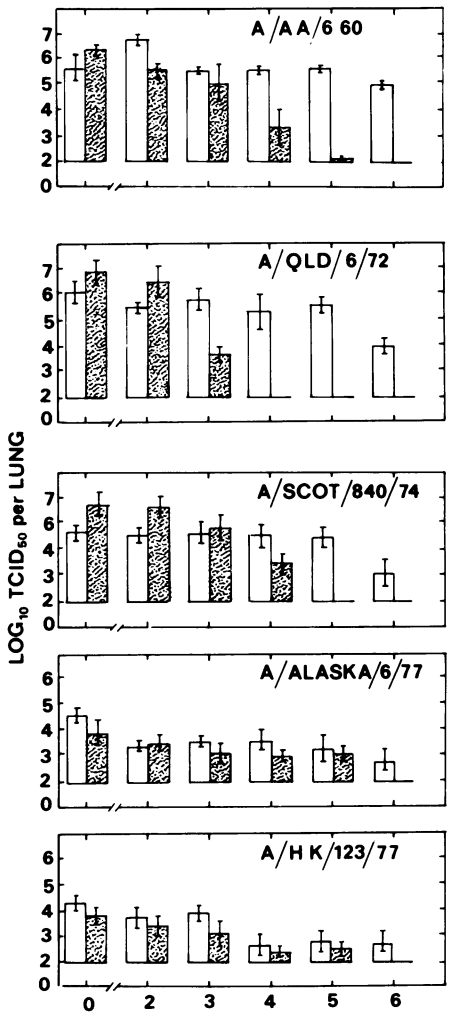

FIG. 1. Immunogenic response of mice to a single intranasal dose of *ca* and parental wild-type viruses described in Table 1. Groups of mice were inoculated with various doses of each *ca* and parental wild-type virus. After 4 weeks each mouse was challenged with $10^{4.5}$ $TCID_{50}$ of wild-type virus with similar surface antigens to the vaccine virus and killed after a further 3 days. Lung extracts were prepared from each animal, and the virus present was determined. The mean ± SEM for five to six animals per group is shown. Open histograms represent yields from mice inoculated with *ca* viruses, and shaded histograms represent yields from mice inoculated with wild-type viruses.

The results in Fig. 1 indicate that intranasal vaccination with cold-adapted recombinants at doses of up to 10^6 $TCID_{50}$ per mouse was insufficient to prevent multiplication of the challenge dose of $10^{4.5}$ $TCID_{50}$ of wild-type viruses with similar surface antigens. When virulent viruses were used to vaccinate the mice, clearance of homologous challenge viruses was obtained at much lower doses. The 50% clearing dose was defined as the infectious dose of virus which was sufficient to prevent detection of virus in 50% of mice after challenge, as determined by the method of Reed and Muench (15). This dose was $10^{3.24}$ $TCID_{50}$ for A/AA/6/60, $10^{2.64}$ for A/Qld/6/72, $10^{3.27}$ for A/Scot/840/74, $10^{4.5}$ for A/Alaska/6/77, and $10^{4.71}$ for A/HK/123/77, which was determined in a separate experiment. By converting the proportion of mice per vaccinating dose which showed complete clearance of challenge virus to logits and applying the model of Nelder and Wedderburn (13), strains A/AA/6/60, A/Qld/6/72, and A/Scot/840/74 were shown to be significantly better immunogens than A/Alaska/6/77 ($P < 0.001$). The result for A/HK/123/77 was obtained in a separate experiment and cannot be directly compared. However, both A/Alaska/6/77 and A/HK/123/77 appear to be poorer immunogens than the other viruses.

The lower immunogenicity of *ca* recombinants is further suggested by an experiment in which groups of mice were vaccinated with 10^5 $TCID_{50}$ of A/Qld/6/72-*ca* and challenged with 10^2 to 10^6 $TCID_{50}$ of wild-type A/Qld/6/72. Complete clearance was achieved only when challenge doses of less than 10^4 $TCID_{50}$ per mouse were used.

Extent of growth of *ca* recombinants and wild-type parental viruses in the respiratory tracts of mice. Differences in immunogenicity between *ca* viruses and their wild-type parent strains in Fig. 1 were considered likely to be due to the restricted growth of *ca* viruses in the respiratory tracts of CSL mice (12). Restriction has been previously demonstrated for A/AA/6/60-*ca* in comparison with a contemporary virulent H2N2 strain (19). The *ca* viruses used in the present study were also examined for evidence of growth restriction. Doses of $10^{4.5}$ $TCID_{50}$ of each *ca* virus and its wild-type parental strain were inoculated by the intranasal route into groups of six mice. After 3 and 4 days, individual mice were sacrificed, and extracts of both lungs and turbinates were prepared for virus assay. The results in Fig. 2 reveal that all *ca* recombinants were restricted in their growth in both lungs and turbinates in comparison with wild-type viruses with similar surface antigens, except for A/AA/6/60, where little turbinate growth occurred. In a similar study with hamsters (17), both *ca* viruses and their wild-type parental virulent strains replicated to a similar extent in turbinates, but the growth of *ca* recombinants in lungs was considerably reduced. The increased growth of the wild-type viruses A/AA/6/60 and A/Qld/6/72 in the lungs of mice, compared with A/Alaska/6/77 and A/HK/123/77, is consistent with the results in Fig. 1 showing that these viruses are better immunogens; A/Scot/840/74 was intermediate in its growth.

Enhancement of immunogenicity for *ca* viruses by administration of two doses. Groups of mice were inoculated intranasally with graded doses of each *ca* virus described in Table 1. After 3 weeks each mouse was reinoculated with an identical dose of the same virus and challenged after a further 3 weeks with $10^{4.5}$ $TCID_{50}$ of a wild-type virus with similar surface antigens. Lung extracts from individual mice were prepared for virus assay after a further 3 days. The results in Fig. 3 indicate that all *ca* viruses, when administered in two identical doses, are efficient immunogens. The calculated dose required to prevent the multiplication of a homologous wild-type challenge virus in 50% of animals was $10^{1.13}$ $TCID_{50}$ for A/AA/6/60-*ca*, $10^{1.17}$ for A/Qld/6/72-*ca*, $10^{2.3}$ for A/Scot/840/74-*ca*, $10^{2.5}$ for A/Alaska/6/77-*ca*, and 10^3 for A/HK/123/77-*ca*. Both A/AA/6/60-*ca* and A/Qld/6/72-*ca* were shown to be significantly better immunogens than the other three *ca* viruses ($P < 0.001$). These differences in immunogenicity must be due to surface antigens, since all *ca* viruses, except A/Scot/840/74-*ca*, have common genes which code for proteins other than surface antigens (1) (Table 1).

Serological responses to one or two doses of *ca* and wild-type viruses. The HI response to each of the *ca* viruses used in this study and to their wild-type parental strains was determined. Groups of 12 mice were inoculated by intranasal route with 10^5 $TCID_{50}$ of each virus. After 3 weeks, six animals per group were given a second dose of the same virus, and all animals were bled after a further 3 weeks. Individual HI titrations were carried out with the same *ca* or

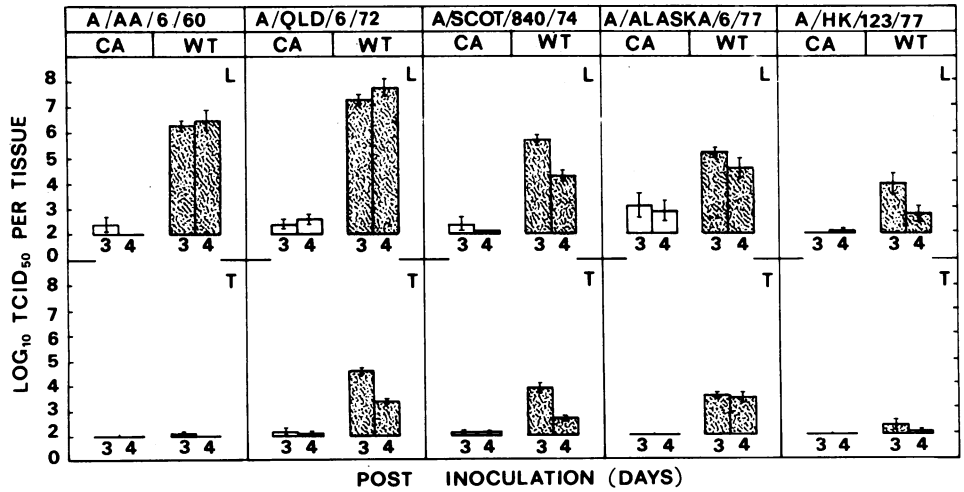

FIG. 2. Virus yields in the respiratory tracts of mice inoculated with *ca* and wild-type viruses containing similar surface antigens. Mice were inoculated with $10^{4.5}$ $TCID_{50}$ of each virus, and the virus present was determined in extracts prepared from lungs (L) and turbinates (T) after 3 and 4 days. The mean ± SEM for five to six animals is shown. Open histograms represent the virus present in mice inoculated with *ca* viruses, and shaded histograms represent yields for wild-type viruses with similar surface antigens.

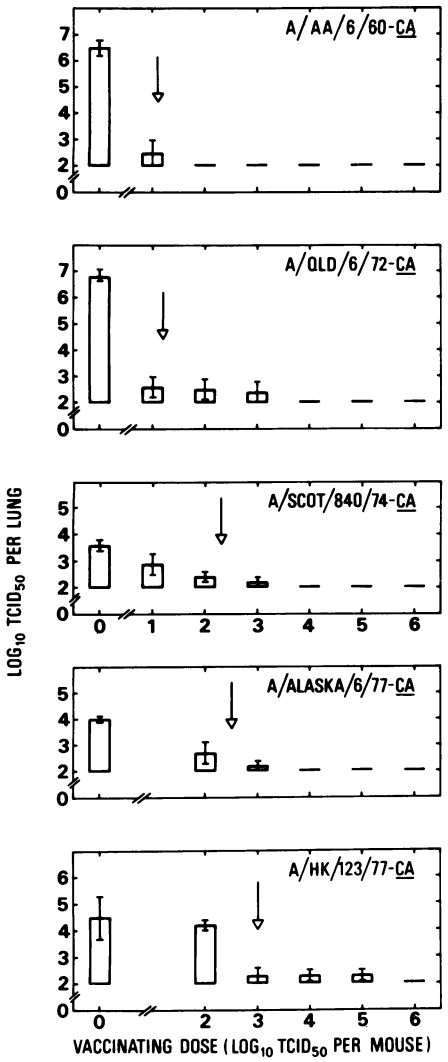

FIG. 3. Immunogenic response of mice to two intranasal doses of *ca* viruses. Groups of mice were inoculated with various doses of each *ca* virus. After 3 weeks, each mouse was reinoculated with an identical dose of the same virus and challenged after a further 3 weeks with $10^{4.5}$ $TCID_{50}$ of a wild-type virus with similar surface antigens. Extracts were prepared from the lungs of each animal after a further 3 days, and the virus present was determined. The mean ± SEM for five to six animals per group is shown. The arrows represent the 50% protective dose of each *ca* virus examined.

wild-type virus used for vaccination; the geometric mean titer for each group is presented in Table 2. The results suggest that (i) much less HI antibody was induced from a single dose of each *ca* virus than its parental wild-type strain; (ii) except for A/Scot/840/74, two doses of each wild-type virus produced an enhancement in titer; the extent of this enhancement varied but did not appear to be as great as when a second dose of each *ca* virus was used; and (iii) the A/HK/123/77 surface antigens of both the *ca* recombinant and the wild-type parent appeared to be relatively inefficient at inducing HI responses.

Although the inoculum of 10^5 $TCID_{50}$ used for all *ca* viruses administered on two occasions was much greater than the 50% clearing dose (Fig. 3), levels of HI antibody induced by different viruses varied considerably and thus do not constitute a reliable index of immunity. Further evidence of the unreliability of HI determinations was obtained from an experiment in which the 50% dose of A/AA/6/60 and A/AA/6/60-*ca* required to induce the production of detectable HI antibody after a single inoculation was shown to be identical (10^4 $TCID_{50}$ per animal).

Clearance of heterologous wild-type challenge strains after vaccination with two doses of A/AA/6/60-*ca*. Mice were inoculated intranasally with two doses of A/AA/6/60-*ca* (10^5 $TCID_{50}$ per animal) at an interval of 3 weeks. A control group was inoculated with PBS. After a further 3 weeks, animals from each group were challenged with $10^{4.5}$ $TCID_{50}$ of a particular wild-type virus, and after a further 3 days, lung extracts were prepared from individual animals for virus assay. The results for two experiments are shown in Fig. 4. In the first experiment (Fig. 4, panel A), a 3.7 to 4.5 $\log_{10}$ $TCID_{50}$ reduction in titer in the lung was noted for both the homologous A/AA/6/60 and the heterologous A/Qld/6/72, A/Vic/3/75, and A/NT/60/68 influenza A challenge viruses (Student's *t* test; $P < 0.01$ for A/NT/60/68; $P < 0.001$ for the others). For B/Singapore/222/79 a reduction of 1.2 $\log_{10}$ $TCID_{50}$ was noted, which was marginally significant ($P < 0.06$). Because egg-derived antigens in both the vaccine and the challenge viruses could have contributed to heterologous cross-protection, a further experiment was carried out in which mice were inoculated with two doses of 10^5 $TCID_{50}$ of egg-grown A/AA/6/60-*ca* and challenged with the H1N1 virus A/HK/123/77 or B/Singapore/222/79 after growth in either eggs or MDCK cell cultures (Fig. 4, panel B). Although protection was noted against the H1N1 viruses grown in either host, the observed reduction in titer for B/Singapore/222/79 when grown in either host was not significant. To further clarify the situation for B/Singapore/222/79, the virus titers of parallel lung extract samples from mice challenged with B/Singapore/222/79 in the second experiment were determined by more accurate plaque assays. The mean titer ± SEM for the egg-grown challenge was 5.01 ± 0.35 $\log_{10}$ PFU per lung for the control and 4.30 ± 0.39 for the vaccinated group; for the MDCK challenge these figures are 3.44 ± 0.31 and 3.11 ± 0.09. Neither of these differences was significant ($P < 0.1$).

Relationships between the hemagglutinin surface antigens of various influenza strains used in the cross-protection experiments were examined by cross-HI tests (Table 3). Substantial differences are apparent, indicating that common determinants, undetectable by HI tests, are responsible for the clearance of influenza A challenge strains of different subtype in Fig. 4.

TABLE 2. HI antibody levels in mice inoculated with one or two doses of *ca* recombinant or its parental wild-type virus

Surface antigen	Titer[a]			
	ca Virus		Wild-type parent	
	One dose	Two doses	One dose	Two doses
A/AA/6/60	64	113	226	359
A/Qld/6/72	71	243	202	226
A/Scot/840/74	71	202	127	113
A/Alaska/6/77	40	211	143	226
A/HK/123/77	40	64	70	90

[a] Each dose consisted of 10^5 $TCID_{50}$ of *ca* or parental virus. Titers were determined against the homologous *ca* or wild-type virus. Each figure represents the geometric mean HI titer for the results from six mice.

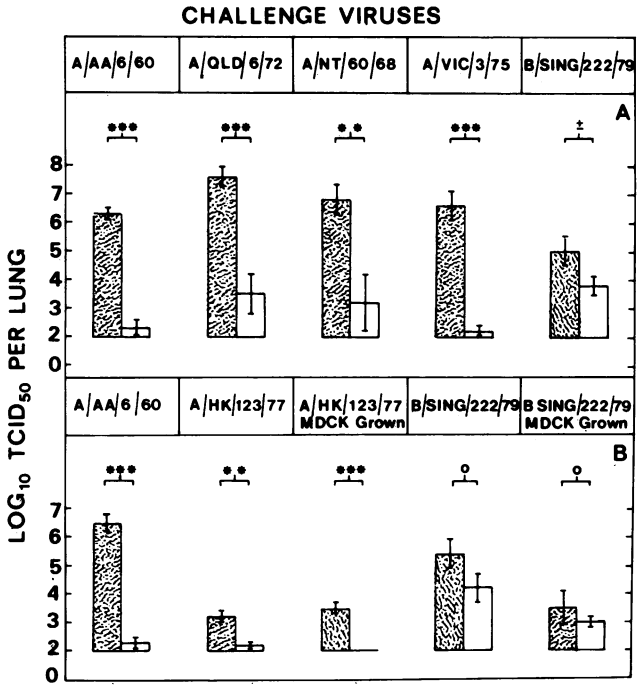

FIG. 4. Immunogenic response of mice to vaccination with two doses consisting of 10^5 $TCID_{50}$ of A/AA/6/60-*ca* and challenge with various wild-type influenza viruses and an influenza B strain. Open histograms with bars represent mean yields ± SEM for vaccinated groups, and shaded histograms with bars represent the mean ± SEM for unvaccinated controls. Two experiments were carried out. In the first (A), both vaccine and challenge viruses were grown in chicken embryos. In the second (B), certain challenge viruses were grown in MDCK cell cultures. Student's *t* test; $P < 0.001$ (***); $P < 0.01$ (**); $0.05 < P < 0.1$ (±).

DISCUSSION

In this study the immunogenicity of several *ca* and wild-type influenza A viruses was determined from the intranasal dose that was sufficient to prevent multiplication of a wild-type challenge in the respiratory tracts of CSL mice. The acquisition of *ca* lesions by several H2N2, H3N2, and H1N1 wild-type viruses greatly reduced their immunogenicity (Fig. 1). This reduction is probably related to the decreased capacity of *ca* viruses to multiply in the respiratory tract (Fig. 2), which has also been observed with inbred CBA/H mice (12), ferrets (7), hamsters (17), and newborn rats (8).

When mice were inoculated with two doses of each *ca* virus at an interval of 3 weeks, solid immunity to the homologous wild-type challenge was observed. The actual dose required on two occasions varied according to the surface antigens of the *ca* virus and was highest for the virus with H1N1 surface antigens and lowest for both A/AA/6/60-*ca* and A/Qld/6/72-*ca*; A/Scot/840/74-*ca* and A/Alaska/6/77-*ca* required intermediate doses to effectively clear the challenge (Fig. 3), and such a relationship is apparent between wild-type viruses with similar surface antigens (Fig. 1). In humans influenzal illness caused by H1N1 viruses has been shown to be relatively mild and the virus isolation rate much lower than that produced by H3N2 viruses (21). A *ca* recombinant vaccine with H1N1 surface antigens also produced a lowered rate of seroconversion and induced virus shedding in slightly fewer volunteers than a *ca* recombinant H3N2 vaccine (20). The present study indicates that the immunogenicity of influenza A viruses for mice is influenced by both the acquisition of *ca* lesions by nonsurface antigen genes and by genes coding for their surface antigens. If these findings apply to humans, the immunizing dose of a particular *ca* recombinant necessary to produce an adequate response in an unprimed individual will have to be varied to take account of its surface antigens. The clearance test described in this study could possibly be used to provide an indication of the vaccinating dose required, relative to that determined in other human trials.

Considerable heterotypic immunity was noted when mice were immunized with two doses, each consisting of 10^5 $TCID_{50}$ of A/AA/6/60-*ca* (H2N2), and challenged with a number of influenza A strains of widely differing serotype (Fig. 4 and Table 3). Similar immunity in the same mouse model has been noted after vaccination by the intranasal route with wild-type influenza A viruses (18), and common relationships have been demonstrated between influenza A viruses from cytotoxic T-cell assays (2, 5).

The above findings suggest that it may be possible to develop highly effective vaccines against influenza A in unprimed humans by the use of low, multiply administered doses of a single *ca* virus. Their effectiveness should not be impaired by minor or even substantial changes in the surface antigens of the epidemic strain of influenza A, and the

TABLE 3. HI relationships between the vaccine virus A/AA/6/60-*ca* and various challenge viruses used in Fig. 4 with antisera prepared from infected mice[a]

Antiserum[b]	H2N2 antigens		H3N2 antigens			H1N1 antigens		B antigens	
	A/AA/6/60-*ca*	A/AA/6/60	A/NT/60/68	A/Qld/6/72	A/Vic/3/75	A/HK/123/77	A/HK/123/77 (MDCK)[c]	B/Sing/222/79	B/Sing/222/79 (MDCK)[c]
A/AA/6/60-*ca*	320	80	<10	<10	<10	<10	<10	<10	<10
A/AA/6/60	226	453	<10	<10	<10	<10	<10	<10	<10
A/NT/60/68	<10	<10	320	113	40	<10	<10	<10	<10
A/Qld/6/72	<10	<10	80	160	80	<10	<10	<10	<10
A/Vic/3/75	<10	<10	40	113	320	<10	<10	<10	<10
A/HK/123/77	<10	<10	<10	<10	<10	57	320	<10	<10
A/HK/123/77 (MDCK)	<10	<10	<10	<10	<10	80	320	<10	<10
B/Sing/222/79	<10	<10	<10	<10	<10	<10	<10	113	80
B/Sing/222/79 (MDCK)	<10	<10	<10	<10	<10	<10	<10	80	80

[a] Results are the geometric mean titers for two sera and replicate titrations.
[b] Sera were obtained from mice 6 weeks after infection with $10^{4.5}$ $TCID_{50}$ of each virus by the intranasal route. All prebleed sera were <10.
[c] Viruses grown in MDCK cell cultures. All others were grown in chicken embryos.

suggested need to continually update the vaccine strain and carry out clinical trials at short notice on each new recombinant (11), a severe deterrent to the development of living vaccines, may not be necessary. There is some epidemiological evidence (3, 4) that earlier priming by "late" H2N2 viruses of the Asian series induces protection against "early" H3N2 viruses.

An earlier study had suggested that some residual virulence for humans was associated with the CR-18 A/Scot/840/74 recombinant at doses in excess of 10^8 $TCID_{50}$ per volunteer (10). A suggested reason for this virulence is the presence of a wild-type parental gene coding for nonstructural protein in this recombinant (10) (Table 1). The present studies do not reveal any immunogenic property of CR-18 that might be responsible for residual virulence, which could be a property of all *ca* recombinants when administered at these doses. If our findings apply to humans, two relatively small doses administered at intervals of 3 to 4 weeks should overcome any problem of residual virulence and produce substantial immunity. The cost of the vaccine would be much less than that of a single, very high dose.

Studies are at present under way to determine the effects of vaccination with *ca* viruses in mice primed with virulent heterotypic viruses in an attempt to mimic the situation in adult humans at times of major antigenic shift.

LITERATURE CITED

1. **Cox, N. J., H. F. Maassab, and A. P. Kendal.** 1979. Comparative studies of wild-type and cold-mutant reassortment of genes during preparation of live virus vaccine candidates by recombination at 25° between recent H3N2 and H1N1 epidemic strains and cold-adapted A/Ann Arbor/6/60. Virology **97**:190–194.
2. **Effros, R. B., P. C. Doherty, W. Gerhard, and J. Gennink.** 1977. Generation of both cross-reactive and virus-specific T cell populations after immunization with serologically distinct influenza A viruses. J. Exp. Med. **146**:557–568.
3. **Gill, P. W., and A. M. Murphy.** 1976. Naturally acquired immunity to influenza type A. Med. J. Aust. **2**:329–333.
4. **Hope-Simpson, R. E.** 1972. Protection against Hong Kong influenza. Br. Med. J. **4**:490–491.
5. **Lin, L.-Y., and B. A. Askonas.** 1980. Cross-reactivity for different type A influenza viruses of a cloned T-killer cell line. Nature (London) **288**:164–165.
6. **Maassab, H. F.** 1969. Biologic and immunologic characteristics of cold-adapted influenza virus. J. Immunol. **102**:728–732.
7. **Maassab, H. F.** 1975. Properties of influenza virus "cold" recombinants, p. 755–763. *In* B. W. J. Mahy and R. D. Barry (ed.), Negative strand viruses, vol. 2. Academic Press, Inc., New York.
8. **Mahmud, M. I. A., H. F. Maassab, R. Jennings, and C. W. Potter.** 1979. Influenza virus infection of newborn rats: virulence of recombinant strains prepared from a cold-adapted, attenuated parent. Arch. Virol. **61**:207–216.
9. **Moritx, A. J., C. Kunz, H. Hofman, E. Liehl, P. Reeve, and H. F. Maassab.** 1980. Studies with a cold-recombinant A/Victoria/3/75 (H3N2) virus. II. Evaluation in adult volunteers. J. Infect. Dis. **142**:857–860.
10. **Murphy, B. R., H. P. Holley, Jr., E. J. Berquist, M. M. Levine, S. B. Spring, H. F. Maassab, A. P. Kendal, and R. M. Chanock.** 1979. Cold-adapted variants of influenza A virus: evaluation in adult seronegative volunteers of A/Scotland/840/74 and A/Victoria/3/75 cold-adapted recombinants derived from the cold-adapted A/Ann Arbor/6/60 strain. Infect. Immun. **23**:253–259.
11. **Murphy, B. R., S. B. Spring, and R. M. Chanock.** 1976. Live vaccine: production and use, p. 179. *In* P. Selby (ed.), Influenza vaccine: virus, vaccines and strategy. Sandoz Institute for Health and Socio-Economic studies and Academic Press, Inc., New York.
12. **Nai-Ki, M., Z. Yong-He, G. L. Ada, and G. A. Tannock.** 1982. Humoral and cellular responses of mice to infection with a cold-adapted influenza A virus variant. Infect. Immun. **38**:218–225.
13. **Nelder, J. A., and R. W. M. Wedderburn.** 1972. Generalized linear models. J. R. Stat. Soc. A **135**:370–384.
14. **Palmer, D. F., W. R. Dowdle, M. T. Coleman, and G. C. Schild.** 1975. Advanced laboratory techniques for influenza diagnosis, p. 25–63. Immunology series no. 6. procedural guide. Centers for Disease Control, Atlanta, Ga.
15. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27**:493–497.
16. **Smith, J. B.** 1979. Survey of laboratory animals being maintained in Australia, p. 1, 3rd revision. John Curtin School of Medical Research, Australian National University, Canberra.
17. **Spring, S. B., H. F. Maassab, A. P. Kendal, B. R. Murphy, and R. M. Chanock.** 1977. Cold adapted variants of influenza A. II. Comparison of the genetic and biological properties of *ts* mutants and recombinants of the cold-adapted A/AA/6/60 strain. Arch. Virol. **55**:233–246.
18. **Tannock, G. A., M. C. Wark, L. E. Smith, and M. M. Sutherland.** 1981. A clearance test in mice using non-adapted viruses to determine the immunogenicity of influenza strains. Arch. Virol. **70**:91–101.
19. **Wark, M. C., G. A. Tannock, M. M. Sutherland, and L. E. Smith.** 1980. A murine model for assessment of living attenuated influenza A vaccines. Aust. J. Exp. Biol. Med. Sci. **58**:615–626.
20. **Wright, P. F., N. Okabe, K. T. McKee, H. F. Maassab, and D. T. Karzon.** 1982. Cold-adapted influenza A recombinant vaccines in seronegative young children. J. Infect. Dis. **146**:71–79.
21. **Wright, P. F., J. Thompson, and D. T. Karzon.** 1980. Differing virulence of H_1N_1 and H_3N_2 influenza strains. Am. J. Epidemiol. **112**:814–819.