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## Selection of neutralizing antibody escape mutants with type A influenza virus HA-specific polyclonal antisera: possible significance for antigenic drift

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### SUMMARY

Ten antisera were produced in rabbits by two or three intravenous injections of inactivated whole influenza type A virions. All contained haemagglutination-inhibition (HI) antibody directed predominantly to an epitope in antigenic site B and, in addition, various amounts of antibodies to an epitope in site A and in site D. The ability of untreated antisera to select neutralization escape mutants was investigated by incubating virus possessing the homologous haemagglutinin with antiserum adjusted to contain anti-B epitope HI titres of 100, 1000 and 10000 HIU/ml. Virus-antiserum mixtures were inoculated into embryonated hen's eggs, and progeny virus examined without further selection. Forty percent of the antisera at a titre of 1000 HIU/ml selected neutralizing antibody escape mutants as defined by their lack of reactivity to Mab HC10 (site B), and unchanged reactivity to other Mabs to site A and site D epitopes. All escape mutant-selecting antisera had a ratio of anti-site B (HC10)-epitope antibody:other antibodies of  $\geq 2.0:1$ . The antiserum with the highest ratio (7.4:1) selected escape mutants in all eggs tested in four different experiments. No antiserum used at a titre of 10000 HIU/ml allowed multiplication of any virus. All antisera used at a titre of 100 HIU/ml permitted virus growth, but this was wild-type (*wt*) virus. We conclude that a predominant epitope-specific antibody response, a titre of  $\geq 1000$  HIU/ml, and a low absolute titre of other antibodies ( $\leq 500$  HIU/ml) are three requirements for the selection of escape mutants. None of the antisera in this study could have selected escape mutants without an appropriate dilution factor, so the occurrence of an escape mutant-selecting antiserum in nature is likely to be a rare event.

### INTRODUCTION

Type A and type B influenza viruses are a major cause of human respiratory disease, and of mortality in the elderly and those with predisposing clinical conditions such as chronic diseases of the heart, lungs and kidneys [1]. Influenza viruses undergo continuous antigenic change of their surface haemagglutinin (HA) and neuraminidase (NA) proteins, so that both natural and vaccine-acquired immunity rapidly be-

come redundant unless vaccination is repeated on an annual basis with the current virus strains. The inactivated vaccine gives 60–90% protection against the homologous strain [2]. Type A and B viruses undergo antigenic changes through the phenomenon of antigenic drift, and type A viruses by antigenic shift [3]. However, most influenza is caused by epidemics resulting from antigenic drift in which the viral surface antigens undergo progressive antigenic changes, so that immunity gained from infection is no longer effective after about 4 years. Both the HA and the NA undergo antigenic drift, but changes in the former are clinically the most significant. Such viruses

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have at least 4 amino acid substitutions in 2 separate antigenic sites of the HA [4].

Current theory holds that antigenic drift is driven by antibody, and that this can be modelled by neutralizing antibody escape mutants in the laboratory. However, there is a problem with this logic because escape mutants only arise in the presence of a single monoclonal antibody (Mab). When two or more Mabs to discrete epitopes are present in equivalent amounts, no escape mutants are selected. This happens because the frequency of one amino acid substitution is about  $10^{-5}$ , and of two is  $10^{-10}$ , and variants are not seen to arise at this rate. Thus one would not expect a polyclonal antiserum to be able to select escape/drift mutants. It has been known for nearly half a century that escape mutants of different viruses can be isolated in the laboratory using immune serum. However, these experiments have all required non-natural conditions, such as highly selected fractions of influenza antiserum [5–8], or low concentrations of foot-and-mouth disease virus [9, 10], or HIV antiserum [11, 12] and usually many sequential passages in the presence of the selecting antibody. *In vivo* escape mutants have arisen when vaccination of cattle with foot-and-mouth disease virus [13] or man with hepatitis B virus [14, 15] or during persistent infection of man or non-human primates by HIV-1 [16–19], resulted in sub-protective immunity. In addition, our recent work has shown that some of the antisera (12%) from mice immunized 2 or 3 times subcutaneously with inactivated whole influenza A virus were able to select escape mutants to an epitope in antigenic site A, and hence appeared to have an epitope-biased HA-specific secondary antibody response. Analysis of antisera from rabbits immunized with the same virus in a variety of routes, formulations and up to three doses showed that the HA epitope specificity in all of many antisera representing the secondary antiserum response of 12 rabbits studied was also biased to a single epitope, but in antigenic site B. The response was not genetically determined because mice of three different haplotypes and rabbits of three different breeds all behaved in the same way [20–22]. The natural situation can be modelled with Mabs, and escape mutants were selected without hindrance provided one Mab predominated, and the other 2 or 3 Mabs in the mixture constituted a small proportion of the total titre [20].

Here we report that nearly half the rabbit antisera that were biased to an epitope in antigenic site B were able to select antibody escape mutants. This ability

depended on both the ratio of these site B epitope-specific antibodies to other antibodies present, and on the absolute titres of all these antibodies.

## MATERIALS AND METHODS

### Viruses

Influenza A/fowl plague virus/Rostock/34 (A/FPV/R: H7N1) and A/fowl plague virus/Dutch/27 (A/FPV/D: H7N7) were plaque-purified and grown in the allantoic cavity of 10-day-old embryonated hen's eggs for 20 h at 35 °C. These viruses have closely related haemagglutinins and unrelated neuraminidases [21]. Virus was purified by differential centrifugation [20]. Infectivity was measured by plaque assay in monolayers of MDCK cells under an agar overlay medium. Haemagglutination and haemagglutination-inhibition (HI) assays used doubling dilutions of virus in 100  $\mu$ l PBS and 2% chicken red blood cells. A 50% end-point was interpolated between complete and no agglutination. HI titres were determined with doubling dilutions of antibody and four haemagglutinating units (HAU) of virus, again by interpolation.

### Preparation of antisera to type A influenza virus

Young adult New Zealand White, English Half-Lop and Chinchilla F1 rabbits were obtained from a number of different suppliers. These were immunized by inoculation of  $5 \times 10^4$  HAU purified A/FPV/D virus into a peripheral ear vein, except for rabbit 3A1 which received A/FPV/R in the same way (Table 1 and [21, 22] for more details). Rabbits 191, 192 and 2A6 received virus inactivated with 0.1%  $\beta$ -propiolactone (Sigma), although A/FPV does not multiply in rabbits. All rabbits received a priming injection plus a variable number of booster (B) injections as indicated in Table 1. Serum was obtained from venous blood from the ear, and was stored without preservative at  $-20$  °C. All animal experiments followed the guidelines laid down by the UK Coordinating Committee for Cancer Research.

### Assay of HA-epitope specificities present in antisera

The HA epitope specificity of antisera was assayed using neutralizing antibody double escape mutants and HI assays [20]. Briefly, double escape mutants were prepared as above from *wt* FPV/R by sequential selection with 2 of 3 FPV/R neutralizing Mabs (HC2, HC10 and HC61). These are directed to epitopes

Table 1. Summary of immunization schedules and titration of the epitope-specific HI titres present in rabbit antisera\*

Rabbit	Antiserum† designation	1/HI titre of antiserum using the escape mutants		
		A + B - D -	A - B + D -	A - B - D +
Chinchilla F1	45 B + 6	9000	100000	4500
New Zealand White	191 BB + 7	13000	100000	9000
New Zealand White	6 B + 6	18000	71000	4500
New Zealand White	192 BB + 14	4500	25000	4500
New Zealand White	192 BB + 7	4500	18000	4500
New Zealand White	192 B + 21	2000	4500	1000
Half-Lop	40 BB + 8	50000	141000	50000
New Zealand White	3A1 BB + 7	4500	9000	2000
Half-Lop	3B4 BB + 5	2000	4000	1000
Half-Lop	2A6 B + 21	1000	3000	2000

\* HIU/ml; see text for an explanation of the rationale of using double neutralization escape mutants for this assay.

† All rabbits were immunized intravenously; the antiserum designation 45 B + 6 is shorthand for: rabbit 45 given a primary inoculation and one booster (B) inoculation, and bled from an ear vein at 6 days after the boost.

which are assumed to correspond with antigenic sites A, B or D respectively of the H3 haemagglutinin [23, 24]. In our short-hand nomenclature these are referred to by the site which has *not* been mutated as a result of Mab selection: e.g. 'A<sup>+</sup>' refers to the escape mutant A<sup>+</sup>B<sup>-</sup>D<sup>-</sup>, which is mutated in the HC10 epitope of site B and the HC61 epitope of site D as a result of selection with the respective Mabs, but not at the HC2 epitope of site A. In this way we also produced 'B<sup>+</sup>' (A<sup>-</sup>B<sup>+</sup>D<sup>-</sup>) and 'D<sup>+</sup>' (A<sup>-</sup>B<sup>-</sup>D<sup>+</sup>) double escape mutants. The mutated sites no longer react in HI or neutralization assays with the cognate Mab.

## RESULTS

### Selection of escape mutants with influenza virus-specific antisera

The HI antibody titres determined with the double escape mutants, are shown in Table 1. All had a response biased to the epitope in antigenic site B that is defined by Mab HC10, but individual antisera varied in titre by up to 33-fold. In addition antisera contained different amounts and different titres to epitopes in sites A and D, but no substantial titres to other epitopes [21, 22; data not shown]. In order to test their ability to select escape mutants, antisera were diluted to contain final anti-B epitope HI titres of 100, 1000 and 10000 HIU/ml. Mixtures of A/FPV/R virus (final concentration 10 HAU/ml or about

10<sup>6</sup> pfu/ml) and antiserum were injected (100 µl) into the allantoic cavity of embryonated hen's eggs and progeny virus examined without further selection. Eighty percent (8/10) of the antisera at a titre of 1000 HIU/ml allowed growth of virus. This was examined by HI with Mabs HC2 (site A), HC10 (site B) and HC61 (site D). According to these tests, 50% (4/8) antisera selected virus that had a ≥ 12-fold reduction in reactivity with Mab HC10 (Table 2), while reactivity with Mabs HC2 (site A) and HC61 (site D) was unchanged (data not shown). However, apart from antiserum WR45, these antisera did not select escape mutants in every egg inoculated, showing that the conditions bordered on the edge of selection.

### Properties of antisera that select escape mutants

All the antisera that selected escape mutants had a ratio of anti-site B epitope antibody:the sum of antibodies to the other epitopes of ≥ 2.0 (Table 2). WR45 B + 6, the antiserum with the highest ratio (7.4), selected escape mutants in every egg tested in four different experiments (data not shown). Within the limits of the available data, the emergence of escape mutants appeared to be proportional to the ratio of antibodies to the site B epitope:the sum of antibodies to the other epitopes. Below a ratio of two, no escape mutants were detected. Preliminary sequencing of the HA gene of an escape mutant

Table 2. Ability of antisera to select escape mutants

Antiserum	Ratio anti-B: anti-A+anti-D HI titres in antiserum*	Anti-B titre (HIU/ml) used to select escape mutants					
		10000	1000	100	100	100	100
45 B+6	7.4	0/3†	na‡	3/3	<b>3/3</b>	3/3	0/3
191 BB+7	4.6	0/3	na	4/4	<b>1/4</b>	3/3	0/3
6 B+6	3.2	0/4	na	1/4	<b>1/4</b>	4/4	0/4
192 BB+14	2.8	nd	nd	0/5	na	4/4	0/4
192 BB+7	2.0	0/3	na	3/3	<b>2/3</b>	3/3	0/3
192 B+21	1.5	nd	nd	4/4	0/4	4/4	0/4
40 BB+8	1.4	0/4	na	4/4	0/4	4/4	0/4
3A1 BB+7	1.4	nd	nd	2/4	0/2	4/4	0/4
3B4 BB+5	1.3	nd	nd	3/3	0/3	3/3	0/3
2A6 B+21	1.0	nd	nd	0/4	na	nd	nd

\* Epitope-specific antibody; data from Table 1.

† First column: number of A/FPV/R virus-positive eggs/number of eggs inoculated.

‡ Second column: number of eggs yielding escape mutants/number of virus-positive eggs; escape mutants are in bold type. Escape mutants are defined by  $\geq 12$ -fold reduced reactivity with Mab HC10 (site B), and unchanged reactivity to Mabs HC2 (site A) and HC61 (site D).

selected by antiserum WR45 B+6 revealed an inferred single amino acid substitution at amino acid 161 in antigenic site B (data not shown), as was found in escape mutants to Mab HC10 [23], and confirmed the immunological data that this antiserum was indeed selecting conventional neutralizing antibody escape mutants in site B. When antisera were used at a titre of 10000 HIU/ml, no virus multiplication was detected, showing that both *wt* and potential escape mutants were neutralized. This was presumably because there were sufficient antibodies present to unchanged epitopes to neutralize the escape mutants. All antisera used at a titre of 100 HIU/ml permitted virus growth, but this was *wt* virus, suggesting that there was insufficient antibody present to neutralize *wt* virus (Table 2).

## DISCUSSION

Data reported here show that nearly half the antisera raised against whole inactivated type A influenza virus are sufficiently biased in their antibody-specificity profile to be able to select escape mutants with a facility comparable to that of a Mab. However this depended on three factors: (i) that HA-specific antibodies were biased to one epitope, (ii) that antibody to the predominant epitope had a titre of  $\geq 1000$  HIU/ml, and (iii) that other antibodies present did not exceed a combined titre of 500 HIU/ml. Thus

in nature, none of the escape mutant selecting antisera above would have selected escape mutants, since all required dilution to achieve the correct balance of antibodies.

There is currently no explanation for the immune system of rabbits not responding with antibody to the many epitopes of the HA that do not react with Mabs HC2, HC10 and HC61, especially as the animals are outbred and of three different strains. None of the epitopes of FPV is blocked by carbohydrate [25], and there was no difference in the glycosylation sites in the strain used in this study (S. Dimes, personal communication). However, our data in mice [20] and those of others on antibody specificity [26–28] and V gene usage [29] show that a narrow HA-specific antibody response can occur. The antiserum response of human hosts to infection with H3 virus to influenza virus HA can also be biased, and this is particularly so in children aged < 5 years [30–33]. Wang and colleagues [33], using a competitive binding assay and ELISA which examined three epitopes of the H3 HA, found a relatively restricted response in adult human sera taken between 1969 and 1971 but not in sera taken in 1978. However, no functional assay to test the ability of these sera to drive antigenic variation was carried out, and the restriction in the HA antibody response found by all these workers was regarded as marginal.

Data reported here demonstrate that the previously

observed epitope-biased antibody response can be significant biologically in the selection of antibody escape mutants *in vitro*. However, it remains to be seen if this situation extrapolates to antibodies resulting from infection in man. If it does, we have for the first time a mechanism to explain antigenic drift which is consistent with laboratory data on antibody escape mutants, i.e. antigenic drift arises through an epitope-biased antibody response. It is not known if escape mutants arise during primary infection, although data from our longitudinal study of rabbits which show a lack of epitope bias in the early immune response suggests this might not be the case [22]. Alternatively, escape mutants could arise during a second infection, if a sterilizing immunity had not been generated. For escape/drift mutants to arise in nature, it is likely that the mucosal antibody response would have to be biased.

If the human antibody response is biased, it seems unlikely that the human population as a whole will be biased to the same single epitope, as significant natural drift variants usually have at least four amino acid substitutions in two different antigenic sites [4]. Thus we would predict that there are at least four different human genetic groupings each of which will have an antibody response biased to a different epitope. Thus when an escape mutant from a person belonging to one genetic group is transmitted to a person of another genetic group, it will become a double escape mutant, and so on until enough amino acid changes have occurred to form a clinically significant drift variant. If human beings do make an epitope-restricted response, we now understand how to prevent antigenic drift arising, as this only occurs when there is a functionally monoclonal antibody response to the HA. What is needed is a vaccine that can stimulate neutralizing antibodies to two or three discrete epitopes, since under these conditions no escape mutants arise [20]. However, there are no data on the epitope specificity of HA antibody stimulated by current human influenza vaccines either in experimental animals or man. Finally the quantitative requirements of the antibody specificities of the antiserum that gives rise to escape mutants (bias to one epitope; a titre of  $\geq 1000$  HIU/ml; and low absolute titre to other epitopes) suggest that only rare individuals can give rise to drift variants. A narrow HA-specific neutralizing antibody response is most likely in people who have only experienced one infection with type A influenza virus, and most of these are children.

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