All rabbits immunized with type A influenza virions have a serum haemagglutination-inhibition antibody response biased to a single epitope in antigenic site B

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Nine rabbits were immunized with type A influenza virions and the epitope specificities of the secondary serum haemagglutination-inhibition (HI) antibody response were analysed with a panel of neutralizing monoclonal (MAb) antibody double escape mutants. Each of the latter was made by sequential selection using a MAb directed to an epitope of a discrete antigenic site, site A, site B or site D, of the haemagglutinin (HA). Thus the epitope reactivity of the escape mutants was represented as A'B'D+, A'B'D- and A'B'D-. The HI antibody response of all antisera was biased to the site B epitope. In 9/12 antisera, obtained from seven rabbits immunized with whole virions, the site B epitope was predominant, representing 65-82% of the total HI antibody. The restriction of HI antibody was unaffected by strain of rabbit, route of inoculation (intravenous or subcutaneous), use of Freund's adjuvant, and up to four immunizing injections. In 3/7 rabbits immunized with whole virus, there was a HI antibody response to the HC2 (site A) or HC10 (site D) epitope, but not both, of equal magnitude to the site B epitope. The HI antibody response in one of the rabbits (#40) became more biased to the site B epitope between the third and fourth immunizing doses. Two further rabbits were immunized with virions which had been partially digested with bromelain and then purified from free HA. Both of these made equal HI antibody responses to the site B epitope and the site D epitope, possibly because their remaining HA spikes were better exposed. Overall, these data demonstrate an unexpected degree of restriction in the production of biologically relevant antibody, such that some rabbits (e.g. #45) mount an HI antibody response which is essentially epitope-specific. Implications for epitope specificity of HI antibody stimulated by human influenza vaccines, and also for the generation of antigenic drift variants are discussed. The reason for the non-responsiveness of the immune system to the many other HI epitopes of the HA is not known.

Introduction

There are 14 subtypes of type A influenza virus (H1–14) which are defined by the antigenicity of their haemagglutinin (HA) protein. Of these, three have caused pandemic and epidemic disease in man. The latter is believed to result from antigenic drift of the viral HA, a phenomenon in which accumulation of amino acid substitutions in key epitopes allows infection of individuals who had previously acquired immunity as the result of infection with the parent virus (Wilson & Cox, 1990; Webster et al., 1992). It is widely believed that antigenic drift is driven by the immune response, and largely by neutralizing antibody specific to the HA. However, in the experimental version of antigenic drift, the production of an antibody escape mutant by a monoclonal antibody (MAb), this is documented as only taking place in the presence of a single MAb (Laver et al., 1979; Yewdell et al., 1979; Webster & Laver, 1980). This is because two independent amino acid substitutions are required if two MAbs to two non-overlapping epitopes are used, and this occurs at the vanishingly low frequency of the product of the individual rates for amino acid substitution (i.e. about $10^{-5} \times 10^{-5} = 10^{-10}$) (Laver et al., 1979; Yewdell et al., 1979; Portner et al., 1980; Lubeck et al., 1980). Thus there is a paradox as to the mechanism of antigenic drift in nature since the antibody response is thought to be too diverse to permit the emergence of drift variants. This belief is based mainly on the knowledge that the HA has up to five antigenic sites each composed of several non-overlapping epitopes, and the assumption that the convalescent neutralizing antibody response would be directed in approximately equal amounts to at least several of these. For these reasons antigenic drift
ought not to take place. However, there is evidence that the human HA-specific antibody response is, under some circumstances and particularly in children, narrower than expected (Haaheim, 1980; Natali et al., 1981; Oxford et al., 1981; Wang et al., 1986). In addition, Lambkin et al. (1994) have recently demonstrated that escape mutants could be selected by mixtures of up to three MAbs (the maximum tested). The first MAb was held at a constant 1000 haemagglutination inhibiting (HI) units/ml and escape mutants emerged when the second and third MAbs were titrated down to 1–3 HIU/ml. Thus it is possible that antigenic drift occurs in nature if at least some antisera are similarly biased to a single epitope. Indeed, this was the implication of the recent work which showed that 12% of antisera from mice given two or three injections of type A influenza virus could select escape mutants in a single passage (Lambkin et al., 1994).

Here we have used neutralizing antibody double escape mutants to examine the epitope specificities present in the HI antibody response of antisera obtained from rabbits immunized with whole type A influenza virions. All antisera had a polyclonal anti-HA response, but all were biased to a single epitope in antigenic site B and, in most antisera, antibody to the site B epitope formed the majority of the total HI antibody response.

**Methods**

*Viruses.* 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 chicken’s eggs for 20 h at 35 °C. Purified virus was prepared by pelleting from allantoic fluid, centrifugation through a 10–40% sucrose density gradient and then banded on a 40–70% sucrose density gradient. A/FPV/R and A/FPV/D have an unrelated neuraminidase and a closely related HA. The HA has 93.8% similarity at the amino acid level (Munk et al., 1992) and is indistinguishable by HI with immune serum and a number of MAbs. There is a one-way cross-reaction at site D presumably as a result of A/FPV/R having amino acid 210ins and A/FPV/D having 210ins. MAbs to site D of the A/FPV/R HA (HC61 and HC58) failed to react with A/FPV/D but antiserum to A/FPV/D reacts in haemagglutination-inhibition with site D of A/FPV/R. Site E of neither strain is masked by carbohydrate (Roberts et al., 1993). Infectivity was measured by plaque assay in monolayers of MDCK cells under an agar overlay medium.

*Monoclonal antibodies.* Mouse MAbs HC2 (site A, amino acid 144), HC10 (site B, amino acid 161) and HC61 (site D, amino acid 210) (Sugrue et al., 1990) were provided by A. R. Douglas and J. J. Skehel (National Institute for Medical Research, London, UK). All are IgG2a. Other H7 MAbs were supplied by R. Rott (1-2 and 1-7: Institut für Virologie der Justus Liebig Universität, Giessen, Germany). MAbs 1-2 and 1-7 do not cross-react by HI with the above epitopes, but have not been mapped more precisely (unpublished data).

*Haemagglutination (HA) and haemagglutination-inhibition (HI) assays.* HA was measured by the agglutination of chicken red blood cells in doubling dilutions of virus in 100 μl PBS and the 50% endpoint interpolated between complete and no agglutination. HI titres were determined with doubling dilutions of antibody and 4 haemagglutinating units (HAU) of virus, again by interpolation of the endpoint.

**Selection of neutralizing antibody double escape mutants by incubation with MAbs.** One ml containing 10⁶ p.f.u. A/FPV/R and 500 HIU of MAb was incubated for 1 h at 25 °C; 100 μl was then inoculated into 10-day-old embryonated chicken’s eggs and allantoic fluid harvested after 48 h incubation at 35 °C. The escape mutant was identified by its failure to react with the selecting MAb, and was plaque-purified twice on MDCK cell monolayers. After growing a stock in eggs, a second MAb selection was made using a mixture of the first MAb and another non-cross-reacting MAb. The double escape mutant was identified by its failure to react with either of the selecting MAbs, and was then plaque-purified as before.

**Immunization of rabbits.** New Zealand White, English Half-Lop and Chinchilla F1 rabbits were obtained from a number of different suppliers. Rabbits were injected with various amounts of purified A/FPV/R and A/FPV/D virus (Table 1) into a peripheral ear vein or subcutaneously with Freund’s adjuvant (Sigma) in multi-site depots over the scapulae. A/FPV does not multiply in rabbits. All rabbits received a priming injection plus a variable number of booster (B) injections. Two rabbits (58 and 59) received virus which had been digested with bromelain (Sigma: Brand & Skehel, 1972). These digested particles, which were separated from free HA by centrifugation through 5% sucrose, had lost most (> 99%) but not all haemagglutinating activity (Taylor, 1986). 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.

**Results**

**Immunization of rabbits with influenza virions**

Nine rabbits, comprising three different strains, were immunized with whole purified FPV/R or FPV/D virions. These were injected intravenously or subcutaneously with Freund’s adjuvant, in the conventional manner as optimized by Webster (1965). Two of the rabbits received virus particles from which most of the HA spikes had been removed by digestion with bromelain. Antiserum was prepared from an ear bleed and stored at −20 °C. All animals received from two to four doses of immunogen (Table 1).

**Analysis of HA-antibody specificities using neutralizing antibody double escape mutants and HI assays**

HI antibody specificities in antisera were analysed using neutralizing antibody double escape mutants. Double escape mutants were prepared from wt FPV/R by sequential selection with two of three FPV/R MAbs directed to antigenic sites A, B or D. 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++' refers to the escape mutant RL13 which is mutated in the HC10 epitope of site B and the HC61 epitope of site D as a result of selection with these MAbs, but not at the
mutant could not be used. Finally, it was decided to use
the individual HI titres to viruses A+, B+ and D+ as a
interaction with the MAbs. Thus this triple escape
increased by over 4-fold compared to
which did not cross-react with the epitopes described
by HI with two other H7-specific MAbs, 1-2 and 1-7,
mutated epitopes. This virus was made and then tested
titre of all HI antibodies which did not react with the
alternative was to prepare a triple antibody-escape
to A+, or B+ or D+, unless one of them is zero. One
antibodies specific to one epitope. Inspection of data in
represents all HI antibody specificities in the antiserum
except those that overlap the epitopes for HC2 (site A)
and HC61 (site D); the titre with B+ virus (RL14)
represents all HI antibody specificities in the antiserum
except those that overlap the epitopes for HC2 (site A)
and HC10 (site B). In the HI assay itself, care was taken
to ensure that exactly the same concentration of HAU of
wt contained little activity to non-A+, B+, D+ epitopes, and
Fig. 1 shows that any subtraction between the HI titres
shows that usually one of these titres was
always low, thus demonstrating that the antiserum
contained little activity to non-A+, B+, D+ epitopes, and
(b) the sum of the HI titres to viruses A+, B+ and D+ was
very close to the HI titre of wt (see below). If there had
been a high titre to non-A+, B+, D+ epitopes, the sum of
HI titres to viruses A+, B+ and D+ should exceed the titre
to wt since the former will be added in three times.

**Table 1. Immunization of rabbits**

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Strain of rabbit</th>
<th>Route of administration</th>
<th>Immunogen</th>
<th>Immunogen dose (HAU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#6</td>
<td>New Zealand White</td>
<td>Intravenous</td>
<td>FPV/D</td>
<td>$5 \times 10^4$</td>
</tr>
<tr>
<td>#26</td>
<td>New Zealand White</td>
<td>Subcutaneous*</td>
<td>FPV/D</td>
<td>$3 \times 10^4$</td>
</tr>
<tr>
<td>#27</td>
<td>New Zealand White</td>
<td>Subcutaneous*</td>
<td>FPV/D†</td>
<td>$3 \times 10^4$</td>
</tr>
<tr>
<td>#36</td>
<td>English Half-Lop</td>
<td>Intravenous</td>
<td>FPV/D</td>
<td>$5 \times 10^4$</td>
</tr>
<tr>
<td>#37</td>
<td>English Half-Lop</td>
<td>Intravenous</td>
<td>FPV/D</td>
<td>$5 \times 10^4$</td>
</tr>
<tr>
<td>#40</td>
<td>English Half-Lop</td>
<td>Intravenous</td>
<td>FPV/D</td>
<td>$5 \times 10^4$</td>
</tr>
<tr>
<td>#45</td>
<td>Chinchilla F1</td>
<td>Intravenous</td>
<td>FPV/R‡</td>
<td>$5 \times 10^4$</td>
</tr>
<tr>
<td>#58</td>
<td>New Zealand White</td>
<td>Intravenous</td>
<td>FPV/R‡</td>
<td>$5 \times 10^4$</td>
</tr>
<tr>
<td>#59</td>
<td>New Zealand White</td>
<td>Intravenous</td>
<td>FPV/R‡</td>
<td>$5 \times 10^4$</td>
</tr>
</tbody>
</table>

* Initial immunization with Freund's complete adjuvant, subsequent immunizations with Freund's incomplete adjuvant; total volume about 0.5 ml.
† Virus was inactivated by incubation with 0.01% formaldehyde overnight.
‡ Rabbit injected with virus particles after partial digestion with bromelain and separated from
free HA by centrifugation.

HC2 epitope of site A. These mutated sites no longer
react in HI with the cognate MAb. Thus the titre with
'wt' represents all HI antibody specificities in the
antiserum to wt FPV/R; the titre with A+ virus (RL13)
represents all HI antibody specificities in the antiserum
except those that overlap the epitopes for HC10 (site B)
and HC61 (site D); the titre with B+ virus (RL14)
represents all HI antibody specificities in the antiserum
except those that overlap the epitopes for HC2 (site A)
and HC61 (site D); the titre with D+ virus (RL9)
represents all HI antibody specificities in the antiserum
except those that overlap the epitopes for HC2 (site A)
and HC10 (site B). In the HI assay itself, care was taken
to ensure that exactly the same concentration of HAU of
wt and double escape mutants was used, and these were
re-checked for every titration, and that the reactive MAb
gave the same HI titre with the double escape mutant as
with wt.

Ideally, we wanted to be able to measure the titre of HI
antibodies specific to one epitope. Inspection of data in
Fig. 1 shows that any subtraction between the HI titres
of A+, B+ or D+ will underestimate the individual HI titre
to A+, or B+ or D+, unless one of them is zero. One
alternative was to prepare a triple antibody-escape
mutant to all three MAbs, so that this would give the
titre of all HI antibodies which did not react with the
mutated epitopes. This virus was made and then tested
by HI with two other H7-specific MAbs, I-2 and I-7,
which did not cross-react with the epitopes described
above or with each other. Unfortunately, the HI titres of
MAbs I-2 and I-7 with the triple escape mutant were
increased by over 4-fold compared to wt (data not
shown), presumably because conformational changes in
the HA trimer of the triple escape mutant facilitated
interaction with the MAbs. Thus this triple escape
mutant could not be used. Finally, it was decided to use
the individual HI titres to viruses A+, B+ and D+ as a
measure of individual antibody specificities because (a)
inspection showed that usually one of these titres was
always low, thus demonstrating that the antiserum
contained little activity to non-A+, B+, D+ epitopes, and
(b) the sum of the HI titres to viruses A+, B+ and D+ was
very close to the HI titre of wt (see below). If there had
been a high titre to non-A+, B+, D+ epitopes, the sum of
HI titres to viruses A+, B+ and D+ should exceed the titre
to wt since the former will be added in three times.

**Distribution of HI antibody specificities in rabbit antisera**

Fig. 1 shows the HI titres obtained with the double
escape mutants A+, B+ and D+, and to wt FPV/R. These
HI titres were summed and compared to the HI titres
obtained with wt virus (data not shown). The summed
escape mutant HI titres of 10/12 (83%) antisera
produced by rabbits immunized with whole (non-
bromelain digested) virus were within 20% of their
respective wt titres, and all were within 40%. Thus from
the argument outlined above, we conclude that there are
few antibody specificities present other than those cross-
reacting with the A+, B+ and D+ epitopes. The percentage
HI titre of each antiserum with each escape mutant is
shown in Table 2. An analysis of their distribution (Fig.
2) shows that most antisera (12/14; 86%) reacted poorly
with A+ virus, with a titre of ≤ 32% of the summed HI
titre; the other antisera from rabbits #40BB+8 and
#26BB+14 had titres of 36% and 44% respectively.
Similarly most antisera (11/14; 79%) reacted poorly
with D+ virus. In contrast, 100% of antisera reacted
strongly with B+ virus. None had a titre of ≤ 32% of the
summed HI titre, 8/14 (57%) had titres of 33–67% of
the summed HI titre, and 6/14 (43%) had titres of >
68% of the summed total.

All rabbits made their maximum HI antibody response
Fig. 1 a–h. For legend see opposite.
Serum HI antibody biased to a single epitope

Fig. 1. Analysis of the distribution of HI antibody specificities in rabbit sera using neutralizing antibody double escape mutants. All rabbits received a priming injection plus a variable number of booster (B) injections. HI assays were conducted using wt A/FPV/R virus and each of the double escape mutants RL13 (A+ B− D−), RL14 (A− B+ D−) and RL9 (A− B− D+). These are mutated in two of three antigenic sites as denoted by the '-' and do not bind the selecting MAb. Only the abbreviated nomenclature (A+, B+, D+) is shown on the x-axis. Pre-immune sera gave HI titres of < 400 HIU/ml.

to the site B epitope, but 4/14 rabbits had an equal HI response to another site: #26BB+ 14 to site A, and #37B+ 5, #58BB+ 7 and #59BB+ 7 to site D. However, rabbits #58 and #59 were immunized with virions which had been partially stripped of HA spikes by digestion with bromelain, and this may have given B cells better access to the antigenic sites of the HA. Thus all antisera have a strong bias of antibody to the HC10 epitope in site B, indicating that this is immunodominant in these rabbits. One of the best examples is the antiserum from rabbit #45 (Fig. 1k) which has nearly 82% of all HI antibody to the site B epitope.

Other aspects relating to antibody specificity

The dominance of the antibody response to the HC10 site B epitope was not decreased by up to four immunizing injections, for example see rabbits #27, #36,
Fig. 2. Analysis of the frequency with which HI antibody specificities directed mainly to a single epitope in antigenic site A, site B or site D occur in rabbit sera. Data from Fig. 1 and Table 2.

Table 2. HI titres of rabbit antisera from each double escape mutant expressed as a percentage of the summed titres of the double escape mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Route of immunization</th>
<th>Sample†</th>
<th>Percentage of the summed titre of the double escape mutants</th>
<th>HI antibody response predominantly to site B epitope</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>A⁺</td>
<td>B⁺</td>
</tr>
<tr>
<td>New Zealand White</td>
<td>i.v.</td>
<td>#6B+8</td>
<td>12</td>
<td>70</td>
</tr>
<tr>
<td>New Zealand White</td>
<td>s.c.</td>
<td>#21BB +14</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td>New Zealand White</td>
<td>s.c.</td>
<td>#27B+14</td>
<td>13</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>#27BB+22</td>
<td>10</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>#27BB+6</td>
<td>12</td>
<td>65</td>
</tr>
<tr>
<td>English Half-Lop</td>
<td>i.v.</td>
<td>#36B+5</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>#36BB+5</td>
<td>22</td>
<td>52</td>
</tr>
<tr>
<td>English Half-Lop</td>
<td>i.v.</td>
<td>#37B+5</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>English Half-Lop</td>
<td>i.v.</td>
<td>#40BB+8</td>
<td>36</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>#40BBB+8</td>
<td>9</td>
<td>73</td>
</tr>
<tr>
<td>Chinchilla F1</td>
<td>i.v.</td>
<td>#45B+5</td>
<td>10</td>
<td>82</td>
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<tr>
<td></td>
<td></td>
<td>#45BB+5</td>
<td>12</td>
<td>70</td>
</tr>
<tr>
<td>New Zealand White</td>
<td>i.v.</td>
<td>#58BB+7</td>
<td>8</td>
<td>46</td>
</tr>
<tr>
<td>New Zealand White</td>
<td>i.v.</td>
<td>#59BB+7</td>
<td>15</td>
<td>42</td>
</tr>
</tbody>
</table>

* i.v., intravenous; s.c., subcutaneous.
† All rabbits received a priming injection plus a variable number of booster (B) injections.

#40 and #45 (Table 2). In particular, the HI titre to B⁺ virus as a percentage of the summed total of HI titres rose from 51% to 73% between the third (#40BB+8) and fourth (#40BBB+8) injections respectively, and this was mainly at the expense of A⁺-specific antibody (Table 2).

The dominance of the HI antibody response to B⁺ virus did not decline with time as shown by antisera taken 14 days and 22 days after the second injection of rabbit #27, and remained between 74% and 77% of the summed total (Table 2). Both of these aspects are currently the subject of a detailed longitudinal investigation.

Table 3 shows that the majority (4/5; 80%) of rabbits immunized intravenously with whole virus gave an HI response which was predominantly to the HC2 epitope of site B. Although only two rabbits were immunized subcutaneously, the fact that one of these produced an HI response predominantly to the HC2 epitope of site B, and did so in the presence of Freund's adjuvant means that the restricted HI response was not unique to intravenous immunization and was not abrogated by the use of adjuvant.

We used three strains of out-bred rabbit which came from different suppliers over a 10 year period. Nonetheless, representatives from all of these were able to
mount a restricted HI antibody response to the HC2 epitope of site B (Table 2).

**Discussion**

We chose to use neutralizing antibody double escape mutants to measure HI antibody specificities because these represented biologically relevant epitopes. The method is not strictly epitope-specific as a double escape mutant, such as A'B'D', which has an unmodified site A epitope and mutations in epitopes in sites B and D, has the potential to react to antibodies to all its other epitopes (called here collectively Z). However, inspection of Fig. 1 shows that there can only be a low level of such antibodies. This conclusion is supported by quantification which showed that the sum of HI antibodies to anti-A + anti-B + anti-D is very close to the HI titre of unmodified wt virus. If a significant level of anti-Z HI antibodies had been present, the summed HI titres should exceed the wt HI titre, since anti-Z antibodies would have reacted approximately equally with each of the three double escape mutants.

It should be stressed that throughout this study we have used conventional immunization procedures. Most rabbits were injected intravenously according to the procedure optimized by Webster (1965). Two others were immunized subcutaneously with Freund's adjuvant. Thus, although we have used only two strains of type A influenza virus, it seems likely that our data are relevant to all previous studies on HI antibody present in rabbit antisera, and it might be appropriate to re-evaluate these in the light of the findings presented here.

All rabbits, regardless of immunization conditions, gave a highly biased HI antibody response, which always included the HC10 site B epitope. No rabbit gave a substantial response to more than two epitopes, and the additional epitope could be either the HC2 site A epitope or the HC61 site D epitope. The restriction did not broaden with an increasing number of immunizing injections and in rabbit #40 became narrower. A narrow HA antibody response in mice was found in regard to both antibody specificity (Smith et al., 1991) and V gene usage (Clarke et al., 1990).

This study poses a number of major questions. (1) What is the mechanism responsible for the lack or relatively poor HI antibody response to the other antigenic sites/non-overlapping epitopes of the HA? Although the H7 HA has not been mapped in detail, it has three antigenic sites which correspond exactly with sites A, B and D of the H3 HA, and seven independent, non-overlapping epitopes [defined by MAbs HC2, HC3, HC10, HC58 and HC61 (Sugrue et al., 1990) and I-2 and I-7 (unpublished data)]. Thus there is no shortage of potentially immunogenic epitopes, but our rabbits were, for unknown reasons, unable to respond significantly to more than two of these. Lambkin et al. (1994) have presumptive evidence that a similar HI antibody restriction occurs also in mice, as 12% of mouse antisera produced by two or three intravenous injections of whole virus were able to select escape mutants in a single passage. However, most of these mouse antisera (10/11) selected escape mutants to the HC2 epitope of site A, and only one to the HC10 epitope of site B. Three of the former were confirmed by sequencing of the HA RNA as having a single amino acid substitution in site A. In addition, rabbit antiserum #45B + 5 was also able, on repeated occasions, to select an escape mutant which mapped to the HC10 epitope of site B (Taylor, 1986; N. J. Dimmock, unpublished data). We have called such antisera which select escape mutants ‘functionally monoclonal’ because effectively only a single MAb has that property. All the rabbit antisera analysed here are currently being tested for their ability to select escape mutants.

(2) Is the HI antibody response similarly restricted when immunologically naive animals are infected with influenza virus? A/FPV does not multiply in rabbits, so mice are currently being infected so that the specificities of their HI antibody response can be determined. It is important to know this in order to understand the situation in man (see 4 below).

(3) Is the HI antibody response as restricted in humans as it is in mice and rabbits? Since all are mammals, it may be that all immunologically naive individuals would behave similarly on immunization. However, this would be difficult to test as only children will have yet to be infected with influenza virus, and few of these receive influenza vaccine. The question of a restricted HA-specific antibody response in man after infection also needs answering. Although some animals are reported to exhibit a broad spectrum of antibody specificities to the

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**Table 3. Comparison of the route of immunization and whether or not the HI antibody response is predominantly to the HC10 epitope of site B**

<table>
<thead>
<tr>
<th>IntraVenous*</th>
<th>Subcutaneous*</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ †</td>
<td>†</td>
</tr>
<tr>
<td>#6B + 8</td>
<td>#37B + 5</td>
</tr>
<tr>
<td>#36B + 5</td>
<td>#58BB + 7★</td>
</tr>
<tr>
<td>(+40BB + 8)</td>
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<td>(#40BB + 8)</td>
<td>#59BB + 7★</td>
</tr>
<tr>
<td>(#45B + 5)</td>
<td>#45BB + 5</td>
</tr>
<tr>
<td>#27B + 14</td>
<td>#26BB + 14</td>
</tr>
<tr>
<td>#27B + 22</td>
<td>#27BB + 6</td>
</tr>
</tbody>
</table>

* All rabbits received a priming injection plus a variable number of booster (B) injections.
† +, Response predominantly to the HC10 epitope of site B; −, response not so restricted.
- Virus particles partially digested with bromelain.
HA of type A influenza virus (Laver & Webster, 1968; Staudt & Gerhard, 1983; Underwood, 1984), data from Haaheim (1980), Natali et al. (1981) and Oxford et al. (1981) suggested that the human anti-H3 response is indeed restricted and particularly more so in children aged ≤ 5 years than in adults. Similarly Wang et al. (1986), using a competitive binding assay and ELISA which examined three epitopes of the H3 HA, found a relatively restricted response in human sera taken between 1969–1971 but not in sera taken in 1978. However, the restriction in the HA antibody response found by all these workers was regarded as marginal, and sera were not subjected to any functional assay to test their ability to drive antigenic variation.

(4) Can the highly restricted ‘functionally monoclonal’ antibody response which we have observed explain the phenomenon of antigenic drift of type A influenza virus in man? No credible mechanism has so far been put forward, since (a) it is known that in practice no escape mutant can arise when virus is put under the immune selection of roughly equal amounts of two neutralizing MAbs directed to non-overlapping epitopes, and (b) it is generally assumed that the neutralizing antibody response will be directed in approximately equal amounts to several different non-overlapping epitopes (Laver et al., 1979; Yewdell et al., 1979; Webster & Laver, 1980). To test this we have first to determine if infection results in the said narrow antibody response. If it does, it seems unlikely that the response in the human population as a whole will be restricted to a single epitope, as significant natural drift variants have four or more amino acid substitutions in two or more of the antigenic sites (Wilson & Cox, 1990). From this we would predict that there are at least two different human genetic groupings which will each have an antibody response biased to a different epitope. Thus when an escape mutant from a person belonging to one genetic group is passed to a person of another genetic group, it will become a double escape mutant with at least two amino acid substitutions. And so this process will continue until enough amino acid changes have occurred for a significant drift variant virus to have been formed.

Were hypothesis to become fact, we are presented with the means to prevent the escape mutants responsible for antigenic drift arising as this only occurs in the presence of a single MAAb alone or of a single MAAb plus ‘low’ concentrations of MAbs to other non-overlapping epitopes (Lambkin et al., 1994). Thus by immunizing with a vaccine which can stimulate two or three neutralizing antibody specificities, escape mutants will only arise at the vanishing low frequency of around $10^{-10}$ or $10^{-15}$ respectively and hence will not appear. However, to the best of our knowledge there is no information about the range of HI antibody specificities stimulated by current human influenza vaccines either in experimental animals or man, and this will have to be evaluated.

It has not escaped our notice that a functionally monoclonal neutralizing antibody response may also be produced by other human pathogens like human immunodeficiency virus type 1 and hepatitis B virus, and may be responsible for the antigenic variants which are produced during infection. (IBV: Carman et al., 1990; Harrison et al., 1991, 1994; Harrison & Zuckerman, 1992. HIV-1: Albert et al., 1990; Arendrup et al., 1992, 1993; Nar a et al., 1990a, b; Tremblay & Wainberg, 1990; Montefiori et al., 1991; Watkins et al., 1993; Schreiber et al., 1994.)

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