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# Heterogeneity of influenza B viruses

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*Antigenic analysis of influenza B strains isolated in 1981–82 from England, Japan, and the USA, using a panel of monoclonal antibodies to the haemagglutinin of B/Oregon/5/80, showed considerable heterogeneity among the isolates, the majority of which had distinct reactivity patterns. Antigenically similar viruses were isolated from England, Japan, and the USA, and heterogeneity was detected among isolates from each country. Further studies are needed to determine whether this marked heterogeneity reflects different co-circulating strains or antigenic drift during an epidemic. The monoclonal antibodies failed to detect any difference between influenza B isolates from patients with Reye's syndrome and those circulating in the community.*

*The monoclonal antibodies, each recognizing different determinants on the haemagglutinin of B/Oregon/5/80, were pooled and compared with ferret antisera to determine if the pool could be used as a reference reagent. The monoclonal antibody pool discriminated between isolates, contained no non-specific inhibitors of haemagglutination, and avoided the problems associated with differences between ferret antisera. In general, viruses that were shown to be antigenically different by ferret antisera were also different with the pooled monoclonal antibodies, but further studies are required to determine the optimal mixture of antibodies that will detect epidemiologically significant differences between strains.*

Antigenic drift in influenza viruses has usually been studied with post-infection ferret antisera or with antisera prepared in chickens, rabbits, or other animals (*1*). Since the antibody response of every animal is different, the results of antigenic analysis of a virus isolate can be expected to vary with the batch of antisera used, and comparisons of viruses between institutions are thus difficult. In addition, most antisera contain inhibitors of influenza virus haemagglutination and, since ferrets are susceptible to infection with influenza viruses, they may have immunological memory of infection with a related strain even if no antibodies are detectable.

An ideal reference antibody reagent would contain high levels of antibodies against all the major antigenic determinants on the surface proteins of the virus and would be available in unlimited supply so that it could be provided to reference centres throughout the world. Theoretically, monoclonal antibody technology could provide such reagents; the antibodies are available in unlimited amounts, have high titres, and are of known specificity. The difficulties are that the preparation of monoclonal antibodies is labour-intensive, and the antibodies are highly specific and not necessarily representative of the major determinants on an antigen.

Monoclonal antibodies have been used to study antigenic drift in influenza A and B viruses (*2–5*), in particular in the haemagglutinin (HA), neuraminidase, and nucleoprotein of influenza A viruses. It has been shown that the haemagglutinin molecule of several influenza A and B strains possesses three or four non-overlapping antigenic areas (*5*, *6*), and that antigenic drift occurs by a series of point mutations that affect one or more of the antigenic regions. Monoclonal antibodies have also provided information on the mechanism of antigenic drift (*7*) and have been useful in locating the probable antigenic regions on the haemagglutinin molecule (*8*, *9*).

In the present study, individual monoclonal antibodies were used to determine the extent of antigenic variation in influenza B viruses isolated during epidemics in England and Japan in 1981–82. The monoclonal antibodies were then pooled and studies carried out to determine if they had any usefulness in assessing significant antigenic drift in field isolates.

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## MATERIALS AND METHODS

### *Viruses*

The following strains of influenza B viruses were used in these studies: Oregon/5/80 (isolated from a case of Reye's syndrome), Singapore/222/79, Singapore/263/79, and various influenza B strains isolated between 1940 and 1982, including a number obtained in England, Japan, and the USA in 1980–82. The viruses were grown in 11-day-old chicken embryos and purified by adsorption to, and elution from, chicken erythrocytes followed by differential centrifugation and sedimentation through a sucrose gradient (10–40% sucrose, 0.5 mol/litre NaCl) (*10*).

### *Serological tests*

Haemagglutination-inhibition (HI) tests were carried out as described previously (*11*). Enzyme-linked immunosorbent assays (ELISA) were done as described by Kida et al. (*12*) using a modification of the method described by Ruitenberg et al. (*13*).

### *Monoclonal antibodies and ferret antisera*

Hybrid cell lines producing antibodies to the HA of B/Oregon/5/80 virus were selected following fusion of myeloma cells SP2/0 Ag14 (*14*) with immune spleen cells, according to the method of Kohler & Milstein (*15*). The spleen cell donor was a BALB/c mouse immunized with one intraperitoneal injection of 3000 HA units of purified B/Oregon/5/80 virus. Fusion was carried out 2–3 months later, 4 days after an intravenous booster injection of antigen (*3*). The culture fluids from the fused cells were screened by ELISA, HI, and neuraminidase-inhibition (NI) tests with inhibitor-resistant influenza viruses for the detection of antibodies. Hybridomas producing antibody were cloned in soft agar and injected intraperitoneally into pristane-treated mice (*1*). Ascitic fluid was collected 7–10 days later and used in the assays. Post-infection ferret sera were prepared as described previously (*16*) and treated with trypsin and periodate to remove non-specific inhibitors.

## RESULTS

### *Specificity of monoclonal antibodies*

The antibodies prepared from the hybridoma lines reacted to high titre in HI tests with B/Oregon/5/80 (Table 1). The specificity of the monoclonal antibodies was also established in radioimmunoprecipitation tests; the antibodies precipitated HA polypeptides of influenza B virus only and did not react with influenza A viruses in serological assays (results not shown). The majority of the 20 monoclonal antibodies did not inhibit influenza B viruses isolated before 1970, although preparation 232/1 did detect a single common determinant between B/Lee/40 and B/Ore/5/80 (Table 1). Of the 20 monoclonal antibody preparations, 15 reacted to high titre with both B/Ore/80 and B/Sing/222/79. In HI tests on more than 50 influenza B strains isolated since 1975, it was shown that the 20 monoclonal antibodies displayed 13 different patterns of reactivity, indicating that most of the antibodies were to different epitopes on the B/Ore/80 virus (results not shown). Monoclonal antibody preparations 163/5 and 280/2 had identical reactivity patterns, as did 107/6, 127/6, and 195/3, suggesting that the antibodies within each group were to the same determinants (Table 1). In subsequent experiments, a single representative from these groups was included.

### *Analysis of recent influenza B isolates*

The panel of monoclonal antibodies to the haemagglutinin of B/Oregon/5/80 was used to study antigenic variability in recent influenza B isolates (Table 2). In 1982, epidemics of influenza B occurred in many countries, including England and Japan. Analysis of recent isolates from these countries showed that some variants reacted with only one or two of the 13 monoclonal antibodies (e.g., B/Eng/19/82 and B/Shiga/75/82) (Table 2), suggesting that antigenic drift had occurred. On the other hand, other isolates from the same epidemic (i.e., B/Eng/123/82) reacted with 10 of the 13 monoclonal antibodies. Preliminary studies suggested that the majority of influenza B viruses isolated in England or Japan in 1982 react with only a few of the monoclonal antibodies, whereas the small number of isolates from the USA that were examined reacted with many more. Further studies are needed to estimate the frequency of isolation of variants that react with only one or two of the monoclonal antibodies in the panel, and to determine whether the severity of an epidemic is related to the number of different co-circulating viruses.

It is apparent that many different antigenic variants of influenza B virus can be isolated during an epidemic year and that antigenically similar viruses are isolated in different countries. The majority of influenza B isolates were distinguishable from each other; 19 isolates from Japan showed 14 patterns of reactivity with the panel of monoclonal antibodies (results not shown). On the other hand, viruses isolated in England and Japan had the same reactivity patterns with the monoclonal antibodies (Table 2).

It is not known whether different strains co-circulate or whether selection of antigenic variants

Table 1. Cross-reactions of monoclonal antibodies to the HA of B/Oregon/5/80 with various influenza B viruses

| Monoclonal antibody | HI antibody titre[a] | | | | |
|---|---|---|---|---|---|
| | Lee/40 | HK/8/73 | Eng/65/76 | Sing/222/79 | Ore/5/80 |
| 152/2 | – | – | – | 150 | 16 000 |
| 113/2 | – | – | 4 800 | 6 080 | 48 000 |
| 134/1 | – | – | 5 440 | 5 760 | 60 800 |
| 146/1 | – | – | 6 400 | 95 | 12 000 |
| 162/1 | – | – | 9 600 | 8 960 | 96 000 |
| 160/1 | – | – | 6 400 | 9 600 | 19 200 |
| 128/2 | – | – | 7 680 | 2 560 | 24 000 |
| 206/2 | – | – | >25 600 | 25 600 | 192 000 |
| 124/4 | – | – | 19 200 | 20 480 | 24 000 |
| 163/5 | – | – | 19 200 | 25 600 | 25 600 |
| 280/2 | – | – | 19 200 | 19 200 | 96 000 |
| 107/6 | – | 600 | > 25 600 | >25 600 | 96 000 |
| 127/6 | – | – | >25 600 | >25 600 | 96 000 |
| 195/3 | – | 100 | >25 600 | >25 600 | 192 000 |
| 238/4 | – | 95 | 25 600 | >25 600 | 96 000 |
| 122/5 | – | – | 1 920 | 2 400 | 3 040 |
| 104/6 | – | – | – | 300 | 4 160 |
| 165/1 | 300 | – | – | 1 280 | 3 200 |
| 232/1 | 6 400 | – | – | – | 2 720 |
| 242/4 | – | – | – | 260 | 1 760 |

[a] Expressed as the reciprocal of the dilution of monoclonal antibody inhibiting 4 HA units of virus. – = no inhibition.

occurs during the course of an epidemic. During an epidemic in Memphis, USA, two distinct antigenic variants were isolated during the same week; B/Mem/1/82 shared 12 of 13 antigenic determinants with B/Ore/5/80, while Mem/3/82 shared only 4 of these determinants. The simplest explanation of these results is that multiple influenza B viruses co-circulate during an epidemic. The similarity between B/Gunma/1/73 and B/Kumamoto/1/81, and between B/Mem/1/82 and B/Albany/1/77, also suggests that antigenic variants may circulate in man for several years or alternatively that similar variants arise at different times from a parental strain.

Since B/Ore/5/80 was isolated from a case of Reye's syndrome, antigenic comparisons were done between this and other influenza B isolates to determine if it possessed any unique determinants on the HA. Monoclonal antibody preparation 152/2 reacted with B/Ore/5/80 only, suggesting that it may be unique (Table 2). However, this monoclonal antibody failed to react with other influenza B isolates from patients with Reye's syndrome (results not shown) indicating that, although it may be antigenically unique, it is not a marker for Reye's syndrome virus.

### *Comparison of ferret antisera and pooled monoclonal antibodies for antigenic analysis of influenza B haemagglutinin*

The approach adopted for the preparation of a reference reagent was to pool as many monoclonal antibodies of different specificity as possible. Each of the 13 monoclonal antibody preparations was assayed by HI and diluted to give an inhibition titre equivalent to the least potent preparation. Ferret antisera were treated with trypsin and periodate to remove non-specific inhibitors (*16*). However, the results (Table 3) showed that inhibitors to some influenza B isolates still remained. Thus, B/Osaka/2/70 was inhibited by pre-immune sera to such high titres that meaningful results could not be obtained with this virus. Mouse ascitic fluid contained no detectable inhibitors of HA at the dilutions used in the assays and the pooled ascitic fluid did not inhibit B/Osaka/2/70 virus, indi-

Table 2. Reactivity of recent influenza B viruses from different geographical regions with monoclonal antibodies to B/Oregon/5/80

| Monoclonal antibodies | Influenza B virus[a] | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2, 3, 4 | 5, 6 | 7 | 8, 9, 10 | 11, 12, 13 | 14 | 15, 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23, 24, 25 |
| 152/2 | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 113/2 | − | − | − | − | − | − | − | − | − | − | + | − | + | − | + |
| 134/1 | − | − | − | − | − | − | − | − | − | − | − | − | − | + | + |
| 146/1 | − | − | − | − | − | − | + | − | + | − | + | − | + | + | + |
| 162/1 | − | − | − | − | − | − | − | − | − | + | + | + | + | + | + |
| 160/1 | − | − | − | − | − | − | − | − | − | − | + | + | + | + | + |
| 128/2 | − | − | − | − | − | − | − | − | − | − | − | − | − | − | + |
| 206/2 | − | − | − | − | − | − | − | − | − | + | − | + | − | + | + |
| 124/4 | − | − | − | − | − | + | − | + | − | + | − | + | + | + | + |
| 280/2 | − | − | − | − | − | − | − | + | − | + | − | + | + | + | + |
| 195/3 | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 238/4 | − | − | − | + | + | − | − | − | + | + | + | + | + | + | + |
| 122/5 | − | − | + | − | + | + | + | + | + | + | + | + | + | + | + |

[a] 1, Osaka/2/70; 2, Ehime/15/81; 3, Okinawa/255/81; 4, Eng/19/82; 5, Osaka/C-5/82;
6, Shiga/75/82; 7, Eng/110/82; 8, Shiga/80/81; 9, Okinawa/271/81; 10, Eng/104/82;
11, Gunma/1/73; 12, Gifu/12/73; 13, Kumamoto/1/81; 14, Ehime/1/82; 15, Vermont/1/77;
16, Memphis/3/82; 17, Kyoto/7/82; 18, Sendai/46/80; 19, Kyoto/2/81; 20, Eng/34/82;
21, Fukuoka/C-27/81; 22, Eng/123/82; 23, Albany/1/77; 24, Memphis/1/82; 25, Memphis/2/82.

− = HI titre more than 10-fold different from that with B/Ore/5/80;
+ = HI titre less than 10-fold different from that with B/Ore/5/80.

Table 3. Comparison of ferret antisera and pooled monoclonal antibodies for antigenic analysis of influenza B viruses[a]

| Virus | No. of reactions with individual monoclonal antibodies[b] | Pre-infection ferret 148 serum | Oregon/80 ferret 148 serum | Pre-infection ferret 149 serum | Oregon/80 ferret 149 serum | Sing/222/79 ferret serum | Oregon/80 monoclonal antibody pool |
|---|---|---|---|---|---|---|---|
| Osaka/2/70 | 0/13 | > 8 | > 8 | > 8 | > 8 | > 8 | – |
| Ehime/15/81 | 1/13 | – | 3.9 | – | 5.4 | 6.3 | 2.7 |
| Shiga/80/81 | 3/13 | – | 3.7 | – | 5.5 | 5.5 | 3.5 |
| Gunma/1/73 | 3/13 | – | 2.6 | 3.0 | 4.4 | 5.5 | 4.0 |
| Kyoto/2/81 | 7/13 | – | 3.6 | – | 4.5 | 4.5 | 3.5 |
| Sendai/46/80 | 7/13 | – | 6.7 | – | 7.5 | ⩾ 8.0 | 6.5 |
| Sing/263/79 | 7/13 | – | 3.4 | – | 5.2 | 6.4 | 5.5 |
| Fukuoka/C-27/81 | 9/13 | – | 5.6 | – | 6.5 | 6.5 | 6.5 |
| Sing/222/79 | 9/13 | – | 5.2 | – | 5.8 | 7.1 | 5.5 |
| Oregon/5/80 | 13/13 | – | 5.5 | – | 6.5 | 6.3 | 6.5 |

[a] HI titres are expressed in $\log_2$ values. Starting dilution = 1/20. – = no inhibition.
[b] See Table 2.

cating that it was antigenically distinct from the other viruses.

In the comparative assays, influenza B viruses that reacted with different numbers of the individual monoclonal antibodies were selected. The results showed that, in general, the viruses that reacted with only a few of the individual monoclonal antibodies also gave the lowest HI titre with the pooled antibodies, and vice versa. Some viruses that reacted with different combinations of individual monoclonal antibodies, e.g., B/Kyoto/2/81 and B/Sendai/46/80, could be distinguished both by the ferret antisera and the pooled monoclonal antibodies, indicating that the pooled monoclonal antibodies are a satisfactory reagent for comparison of strains.

Analysis of the influenza viruses with two different ferret antisera to B/Ore/5/80 and one to B/Sing/222/79 (Table 3) showed differences between the ferret antisera. Ferret antiserum 148 to B/Ore/5/80 was more discriminating, showing an 8-fold difference between B/Gunma/1/73 and B/Ore/5/80, whereas ferret serum 149 gave about a 4-fold difference. The pool of monoclonal antibodies gave a 6-fold difference between these viruses.

Comparative analysis of influenza B viruses with the pool of monoclonal antibodies and with ferret antisera (Table 3) showed that, although the monoclonal antibodies were sometimes more discriminating, in general they gave results similar to those obtained with ferret antisera. The greatest difference with the monoclonal antibody pool was between B/Ehime/15/81 and B/Ore/5/80, where a difference of approximately 10-fold was detected; the most discriminating ferret antiserum showed only a 3-fold difference between these viruses.

## DISCUSSION

Antigenic analysis of influenza B strains isolated in 1981–82, using a panel of monoclonal antibodies to the haemagglutinin of B/Oregon/5/80, indicated that many antigenically distinguishable influenza viruses circulate in an epidemic year and that multiple different strains may co-circulate during an epidemic. Further detailed studies are required on influenza B viruses isolated during the course of a circumscribed epidemic to determine how many different B viruses can co-circulate and whether the different variants arise by selection during the course of the epidemic. These two parameters will be difficult to separate but the isolation of different variants at the beginning of an epidemic should provide information on this point.

Comparative studies, using post-infection ferret antisera and a pool of monoclonal antibodies to the haemagglutinin of B/Ore/5/80 to determine the extent of antigenic diversity in a group of recent influenza B isolates, showed that there are advantages and disadvantages associated with both reagents. As expected, different ferret antisera show different reactivities, and non-specific inhibitors to some strains could not be eliminated, even on treatment with trypsin and periodate. The monoclonal antibodies did

not have these problems but showed greater antigenic differences between some isolates than did the ferret antisera. The studies showed that all the recent influenza B isolates examined could be characterized with the pool of monoclonal antibodies, indicating that the antibodies may have some utility as standard reference reagents. Further studies are necessary with the pooled antibodies to establish what constitutes an epidemiologically significant difference between strains.

It is planned to add more monoclonal antibodies to different antigenic determinants to the pool. Other features affecting the reactivity of the pool are (1) the affinity of the individual monoclonal antibodies and (2) the number of monoclonal antibodies to the different antigenic regions on the haemagglutinin. Each of these parameters should be taken into account in future work.

One of the problems to be considered in the use of pooled monoclonal antibodies for analysis of influenza viruses is the extensive antigenic variation in the haemagglutinin of these viruses, which makes it necessary to prepare new pools of antibodies every few years. Since antigenic drift in influenza B viruses is less extensive than in influenza A viruses (*5*, *17*, *18*), it might be anticipated that a pool of monoclonal antibodies to influenza B haemagglutinin would be useful for a longer period than a similar pool for influenza A viruses. The above studies have shown that the pool of monoclonal antibodies to the haemagglutinin of an influenza B strain from 1980 is useful for antigenic analysis of strains isolated between the early 1970s and 1982. In contrast, a panel of monoclonal antibodies to the haemagglutinin of A/Mem/1/71 (H3N2) reacted with few H3N2 viruses isolated after 1972 (*19*).

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## RÉSUMÉ

### HÉTÉROGÉNÉITÉ DES VIRUS GRIPPAUX B

En vue d'étudier les variations antigéniques des virus grippaux de type B on a préparé des anticorps monoclonaux dirigés contre les différents déterminants de l'hémagglutinine de B/Oregon/5/80. Les différents anticorps monoclonaux ont réagi avec les virus grippaux de type B isolés entre 1970 et 1982. Les isolements grippaux de type B provenant d'Angleterre, du Japon et des Etats-Unis en 1981–1982 étaient extrêmement hétérogènes, réagissant très diversement aux anticorps monoclonaux de la série. Certains isolements ont réagi avec un seul des 13 anticorps monoclonaux, tandis que d'autres ont réagi avec les 12 autres. Des virus analogues du point de vue antigénique ont été isolés en Angleterre, au Japon et aux Etats-Unis en 1981–1982 et l'on a constaté que ces isolements étaient hétérogènes. D'autres études seront nécessaires pour déterminer si cette hétérogénéité prononcée des virus grippaux de type B est due au fait que différentes souches circulaient simultanément ou au glissement antigénique se produisant au cours d'une épidémie.

Le mélange d'anticorps monoclonaux a permis vue meilleur mettre différenciation révélant une plus grande différence antigénique entre les virus que les sérums de furet post-infectieux en IH. En général, les virus différenciés par le mélange d'anticorps monoclonaux l'étaient également par les anti-sérums de furet. Les différents immunsérums de furet ne réagissaient pas de façon uniforme avec des virus grippaux de type B et les inhibiteurs non spécifiques de l'hémagglutinine de certaines souches n'ont pu être éliminés après un traitement à la trypsine et au periodate. En revanche le mélange de liquides ascitiques contenant les anticorps monoclonaux ne présentait aucun inhibiteur non spécifique. Ces études indiquent que les mélanges d'anticorps monoclonaux peuvent avoir quelque utilité comme réactifs de référence, mais d'autres travaux sont nécessaires pour établir quel est le mélange optimal d'anticorps monoclonaux capable de mettre en évidence des différences entre les souches importantes sur le plan épidémiologique. Les anticorps monoclonaux n'ont pu différencier les souches grippales de type B isolées chez des malades présentant le syndrome de Reye et celles en circulation au sein de la population.

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