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# Antigenic Variation of HVJ (Sendai Virus) HN Glycoprotein Detectable by Monoclonal Antibodies during Persistent Infection

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SUMMARY

Three newly established monoclonal hybridoma antibodies to the haemagglutinin molecule of HVJ, designated A7, B3 and F11, recognize operationally non-overlapping antigenic determinants and have neutralizing activity. Using these antibodies, the frequencies of occurrence of neutralization-resistant antigenic variants were analysed in virus populations released from four cell lines persistently infected with HVJ, namely GM2-HVJ, LLCMK2-HVJ, Vero-HVJ and GEsl-HVJ at various passage stages. Antigenic variants were selected from culture fluids of these HVJ carrier cells at a total frequency of $10^{-3.3}$, $10^{-3.8}$ and $10^{-3.6}$ by monoclonal antibodies A7, B3 and F11, respectively. These values were considerably higher than those of $10^{-4.7}$ to $10^{-5.2}$ detected in a stock preparation of wild-type virus with these antibodies. All the variant viruses isolated as above were negative in neutralization, haemagglutination inhibition and immunofluorescent staining tests with each monoclonal antibody used for their isolation, but were positive with the other antibodies.

Previous reports from this and other laboratories have shown that temperature-sensitive (*ts*) mutants, some of which contain altered M (membrane) or P (polymerase) proteins as well as protease activation mutants could be isolated from HVJ persistently infected cell lines demonstrating the selective advantage of these *ts* mutants *in vitro* over the original wild-type virus (Yoshida *et al.*, 1979; Kimura *et al.*, 1979; Ogura *et al.*, 1981, 1982; Sato *et al.*, 1981). Holland and co-workers, using vesicular stomatitis virus (VSV) have shown that genome changes can accumulate during persistent infection (Holland *et al.*, 1979). Ter Meulen and co-workers have also demonstrated the occurrence of antigenic changes detectable by monoclonal antibodies within the H polypeptide of subacute sclerosing panencephalitis (SSPE) virus during the course of a non-productive, persistent infection *in vitro* (ter Meulen *et al.*, 1981). Monoclonal antibodies are highly specific reagents capable of detecting a single amino acid change in a determinant, and have been used to detect neutralization-resistant antigenic variants of HVJ, VSV and influenza A viruses spontaneously occurring at a frequency of approximately $10^{-5}$ (Yewdell *et al.*, 1979; Laver *et al.*, 1979; Portner *et al.*, 1980). Monoclonal antibodies against HVJ glycoproteins have recently been described by several groups (Yewdell & Gerhard, 1982; Portner, 1981; Örvell & Grandien, 1982).

In the present study, we have used three monoclonal antibodies which recognize antigenic determinants that are operationally non-overlapping and have higher neutralizing capability, to estimate the frequency of variation in the antigenic determinants of the haemagglutinin (HN) molecule of HVJ during persistent infection *in vitro*.

Hybridoma cell lines were produced by the methods of Gerhard *et al.* (1980). Cell lines secreting virus-specific antibodies were identified by indirect immunofluorescence staining with fluorescein-conjugated anti-mouse IgG (Miles Laboratories) on acetone-fixed HVJ-infected LLCMK2 cells in a micro-slide culture chamber (Bellco Glass, Inc). Antibody-secreting hybrids were cloned and grown in the peritoneal cavity of BALB/c mice to produce ascites. Monoclonal hybridoma antibodies were initially screened for neutralizing activity

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Table 1. *Haemagglutination inhibiting (HI) and neutralization (NT) activity on monoclonal antibodies*

| Monoclonal antibody | HI* | + Anti-mouse IgG serum | NT† | + Anti-mouse IgG serum |
|---|---|---|---|---|
| A7 | 1280 | 1280 | 1000 | 1000 |
| B3 | 640 | 2560 | 2500 | 5000 |
| F11 | 1280 | 2560 | 2500 | 5000 |

* The HI test was performed by making twofold monoclonal antibody dilutions in microtitre plates and then adding 8 HA units of HVJ. After the mixture had been incubated for 30 min at 4 °C, 25 μl of anti-mouse IgG serum (1/20 dilution) (Fujizoki Corp., Japan) or PBS was added and incubated for a further 30 min, and then 50 μl of 0·5% chicken erythrocytes was added. Maximum agglutination titres were reached after 1 h at 4 °C. Titres are expressed as the reciprocal of the highest twofold dilution causing complete inhibition of haemagglutination by 8 HA units of virus.

† NT tests were done by incubating HVJ with an infectivity titre of 100 p.f.u. with twofold serial dilutions of monoclonal antibodies at 4 °C for 2 h and a further 1 h in the presence or absence of anti-mouse IgG serum (2 μl). The remaining infectivities of the mixtures were assayed on LLCMK2 cells as described previously (Ogura *et al.*, 1981).

against plaque-purified wild-type HVJ. After several cell fusion experiments, three out of 34 hybridoma cell cultures secreting antibodies against HVJ HN protein were selected which could neutralize a large amount of virus completely apart from residual neutralization-resistant antigenic variants. Antibody specificity was determined by radioimmunoprecipitation of [$^{35}$S]methionine-labelled virus particles and SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). These three hybridoma antibodies precipitated only a single polypeptide with the expected mol. wt. of the HN (data not shown).

The results of haemagglutination inhibition (HI) and neutralization (NT) tests using these monoclonal antibodies are shown in Table 1. The monoclonal antibodies A7, B3 and F11 effectively inhibited viral haemagglutinating activity and infectivity. The subsequent addition of anti-mouse IgG serum enhanced HI or NT titres of antibodies B3 and F11 by two- to fourfold. The NT activity of these clones almost exceeded their HI activity.

The frequency of neutralization-resistant antigenic variants present in cloned HVJ wild-type was determined by comparison of the titre of virus treated with monoclonal antibody to the titre of untreated virus ($5 \times 10^6$ p.f.u.). As shown in Table 2, the frequency of non-neutralized virus detected by antibody A7 was not greatly different at 5- to 25-, and at 5- to 125-fold dilution in the absence and presence of anti-mouse IgG serum, respectively. The monoclonal antibody B3 neutralized effectively at 5-fold, and at 5- to 125-fold dilution in the absence and presence of anti-mouse IgG serum, respectively. Antibody F11 showed effective activity at fivefold dilution, with or without anti-mouse IgG serum. When non-neutralized viruses were examined by NT, HI and immunofluorescent staining tests, all the viruses isolated at the frequencies of less than $10^{-4\cdot7}$ were resistant to neutralization by the antibody used to isolate them but neutralized by the other antibodies; this suggests that antigenic determinants recognized by these three monoclonal antibodies were operationally non-overlapping. However, no variant viruses of this type were found among the viruses isolated at a frequency above $10^{-3\cdot7}$. These data indicate that the frequency of neutralization-resistant antigenic variants in the wild-type stock virus was $10^{-4\cdot7}$ to $10^{-5\cdot2}$ irrespective of which of the monoclonal antibodies was employed.

Using these monoclonal antibodies, neutralization-resistant antigenic variants were isolated from HVJ-carrying cells. Four carrier cultures, GM2 (from a 3-methylcholanthrene-induced fibrosarcoma of an inbred golden hamster)-HVJ, LLCMK2-HVJ, Vero-HVJ and GEs1 (from the embryo of an inbred golden hamster)-HVJ cells were recently established after infecting with wild-type HVJ purified by plaque isolation at 39 °C and maintained at 34 °C (Ogura *et al.*, 1981). Almost all of these carrier cells exhibited virus antigens, as judged by immunofluorescence. The result of isolation of neutralization-resistant antigenic variants from culture fluids of four HVJ-carrier cell lines at various passage stages are shown in Table 3. The frequencies of antigenic variants detected in these four carrier cells by monoclonal antibodies were not very different. A total of 59, 21 and 30 clones of variants were isolated from $1\cdot3 \times 10^5$ p.f.u. of virus

Table 2. *Frequency of neutralization-resistant antigenic variants from HVJ wild-type stock detected by monoclonal antibodies**

| Monoclonal antibody | Dilution (-fold) | Frequency of non-neutralized viruses | + Anti-mouse IgG serum |
|---|---|---|---|
| A7 | 5 | $10^{-5.1}$ | $10^{-5.1}$ |
| | 25 | $10^{-4.7}$ | $10^{-5.2}$ |
| | 125 | $>10^{-3.7}$ | $10^{-4.9}$ |
| | 625 | $>10^{-3.7}$ | $>10^{-3.7}$ |
| B3 | 5 | $10^{-4.8}$ | $10^{-4.7}$ |
| | 25 | $>10^{-3.7}$ | $10^{-4.8}$ |
| | 125 | $>10^{-3.7}$ | $10^{-4.9}$ |
| | 625 | $>10^{-3.7}$ | $>10^{-3.7}$ |
| F11 | 5 | $10^{-4.8}$ | $10^{-4.8}$ |
| | 25 | $>10^{-3.7}$ | $>10^{-3.7}$ |

* Neutralization-resistant antigenic variants were isolated from HVJ wild-type stock which had been purified through plaque isolation at 39 °C and grown in the egg allantoic cavity. Fivefold serial dilutions of monoclonal antibodies (20 µl) were mixed with 80 µl of HVJ with an infectivity titre of $5 \times 10^6$ p.f.u. After incubating the mixture for 16 h at 4 °C, 2 µl of anti-mouse IgG serum or Eagle's minimum essential medium was added and the mixture incubated further for 2 h at 4 °C. The infectivities of the mixtures were assayed on LLCMK2 cells as described previously (Ogura *et al.*, 1981).

Table 3. *Isolation of neutralization-resistant antigenic variants from HVJ carrier cells with monoclonal antibodies**

| Cells | Passage no. | Culture fluid p.f.u./0·2 ml | No. of variants isolated with antibody per 0·2 ml: A7 | B3 | F11 |
|---|---|---|---|---|---|
| GM2-HVJ | 15 | $2{\cdot}6 \times 10^3$ | ND† | ND | ND |
| | 17 | $7{\cdot}0 \times 10^3$ | 6 | 1 | ND |
| | 40 | $6{\cdot}0 \times 10^3$ | 4 | ND | ND |
| | 63 | $4{\cdot}4 \times 10^3$ | ND | ND | ND |
| | 205 | $4{\cdot}0 \times 10^3$ | ND | ND | 2 |
| Vero-HVJ | 5 | $1{\cdot}8 \times 10^1$ | 1 | ND | ND |
| | 10 | $<10$ | 1 | ND | ND |
| | 15 | $1{\cdot}9 \times 10^1$ | ND | ND | ND |
| | 30 | $3{\cdot}6 \times 10^1$ | 1 | ND | ND |
| | 40 | $<10$ | 2 | ND | ND |
| | 60 | $1{\cdot}1 \times 10^1$ | ND | ND | ND |
| LLCMK2-HVJ | 12 | $7{\cdot}4 \times 10^3$ | 1 | 1 | 5 |
| | 20 | $8{\cdot}2 \times 10^4$ | 35 | 17 | 11 |
| | 30 | $4{\cdot}2 \times 10^3$ | 3 | ND | 1 |
| | 50 | $1{\cdot}1 \times 10^1$ | 1 | 1 | ND |
| GEs1-HVJ | 5 | $1{\cdot}1 \times 10^4$ | ND | 1 | 10 |
| | 10 | $1{\cdot}4 \times 10^3$ | ND | ND | ND |
| | 15 | $2{\cdot}2 \times 10^2$ | 1 | ND | ND |
| | 20 | $2{\cdot}4 \times 10^1$ | ND | ND | ND |
| | 50 | $<10$ | 2 | ND | 1 |
| Total | | $1{\cdot}3 \times 10^5$ | 59 | 21 | 30 |
| Average frequency | | | $10^{-3.3}$ | $10^{-3.8}$ | $10^{-3.5}$ |

* Confluent monolayers of each carrier culture were maintained in MEM without calf serum at 34 °C for 24 h and culture fluids were harvested for the isolation of variant viruses. Viruses in the culture fluids were activated by incubating with 1 µg/ml of *N*-acetyl trypsin (Sigma) for 10 min at room temperature and then phenylmethylsulphonyl fluoride (10 µg/ml; Sigma) was added to stop the trypsin action. An aliquot of 50 µl of monoclonal antibody was added to 0·2 ml of culture fluid pretreated with trypsin and incubated for 16 h at 4 °C. Non-neutralized virus was titrated on LLCMK2 cells after further incubation with anti-mouse IgG sera for 2 h.

† ND, Not detected.

from HVJ-carrier cultures by selection with monoclonal antibodies A7, B3 and F11. The frequencies calculated from the above data were $10^{-3.3}$, $10^{-3.8}$ and $10^{-3.5}$, respectively. LLCMK2-HVJ cells at passage 20 produced about half the antigenic variants obtained in the present experiments. Antigenic variants selected from the culture fluid of LLCMK2-HVJ at passage 20 with antibodies A7, B3 and F11 occurred with a frequency of $10^{-3.4}$, $10^{-3.7}$ and $10^{-3.9}$, respectively. Every isolated virus clone was examined by neutralization, haemagglutination inhibition and immunofluorescent staining tests. No neutralization was observed with the monoclonal antibodies used for selection, whereas positive neutralization was observed with other antibodies (data not shown). The above frequencies were considerably higher than the frequencies of $10^{-4.7}$ to $10^{-5.2}$ detected in a stock preparation of HVJ wild-type. The frequencies of antigenic variants detected with monoclonal antibodies directed towards the haemagglutinin of HVJ or influenza A virus and the surface glycoprotein of VSV were of approximately the same order, namely $10^{-5}$ for the three different viruses (Yewdell *et al.*, 1979; Laver *et al.*, 1979; Portner *et al.*, 1980). Ter Meulen and co-workers reported that SSPE virus carrier cells became non-reactive with four out of six monoclonal antibodies against the H polypeptide of SSPE virus when tested by fluorescent antibody staining during the course of a non-productive, persistent infection *in vitro* (ter Meulen *et al.*, 1981). However, they established SSPE virus persistent infection initially by intracerebral inoculation of hamsters with virus and then Vero cells were infected by co-cultivation with brain tissue from these animals. So, in their experiments, there is a possibility that spontaneously occurring mutant viruses were selected by the immune system. Schild *et al.* (1983) demonstrated host-cell selection of influenza virus antigenic variants. In our HVJ persistently infected cell lines, the appearance of antigenic variants was rather random and, as is shown in Table 3, accumulation of antigenic changes was not observed. These results suggest that variations in these antigenic determinants did not lead to selective advantage of viruses during persistent infection *in vitro*. It is still unclear how and why genome changes or mutations leading to variations in antigenicity, cytopathogenicity, temperature sensitivity and so on, some of which might seem to be advantageous, arise at higher frequencies during persistent infection *in vitro*. Youngner *et al.*, using VSV, reported that treatment of host cells with actinomycin D enhanced the frequency of occurrence of *ts* mutants, suggesting that a host cell factor(s) involved in the fidelity of RNA transcription might be repressed by the antibiotic, permitting many errors to be made (Youngner *et al.*, 1981). In our preliminary experiments, attempts to enhance the frequency of antigenic variation by actinomycin D treatment and u.v.-irradiation of host cells were not successful. However, monoclonal antibodies are highly specific reagents capable of detecting a single amino acid change in a determinant (Laver *et al.*, 1979) and their use may be a useful technique to elucidate the host cell factor(s) involved in virus variation during persistent infection *in vitro*.

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