Selection of unique antigenic variants of Newcastle disease virus with neutralizing monoclonal antibodies and anti-immunoglobulin

(neutralization/persistent fraction/sensitization)

RONALD M. IORIO AND MICHAEL A. BRATT

Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, MA 01605

Communicated by Mahlon Hoagland, June 17, 1985

ABSTRACT Monoclonal antibodies were used to isolate nonneutralizable antigenic variants in the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus. It had been found that a large percentage of virus retains infectivity despite binding neutralizing antibody. This high persistent fraction of nonneutralized virus precluded the isolation of variants by the standard treatment with antibody alone. Rabbit anti-mouse immunoglobulin was used to reduce the percentage of virus that remains infectious despite the presence of bound antibody. This procedure made possible the isolation of variants of two distinct types: classical variants, not neutralized because they do not bind the antibody used to select them; and unique variants that, although still capable of binding the selecting antibody, are only slightly neutralized. The general applicability of this method for the isolation of antigenic variants in nonneutralizing epitopes is also discussed.

Analyses of antigenic variants selected to escape neutralization by monoclonal antibodies have contributed greatly to our understanding of hemagglutinin structure and its antigenic variation in influenza viruses (1–6). They also have provided considerable insight into protein structure–function relationships for other viruses, including the G glycoprotein of rhabdoviruses (7, 8) and the hemagglutinin-neuraminidase glycoprotein (HN) of parainfluenza viruses (9, 10) and Newcastle disease virus (NDV) (11). In each case, neutralization-resistant variants were isolated easily from plaques formed after infection by monoclonal antibody-treated virus stocks. The ease of selecting these variants is attributable to the reduction of infectivity by the selecting antibody to a level comparable to that of spontaneously appearing variants in the population.

We have prepared a panel of monoclonal antibodies (designated HN1–HN4) specific for the HN of NDV and used them to topologically define four antigenic sites on the glycoprotein by competition antibody binding assays and additive neutralization assays (12). These assays define broad antigenic areas of the molecule that potentially can be subdivided into epitopes by analysis of nonneutralizable antigenic variants; two antibodies that compete reciprocally for binding to HN may recognize precisely the same epitope, or distinct and perhaps widely separated epitopes, within the topologically defined antigenic site. To construct a fine epitope map for HN, we wanted to select antigenic variants that escape neutralization by each monoclonal antibody. However, our panel of antibodies possesses unusual neutralizing characteristics (13) that preclude the isolation of variants by direct selection as described above: (i) neutralization by each antibody results in a persistent fraction of nonneutralized virus of >1%, but relatively constant for antibodies to a given epitope; (ii) antibodies to all four sites are

required for neutralization to a level comparable to that obtained with polyclonal mouse anti-NDV serum; and (iii) addition of rabbit anti-mouse Ig to the persistent fraction reduces infectivity by an additional 2–3 orders of magnitude, suggesting that most virus particles in the monoclonal antibody-treated population have antibody associated with them. The latter property provides an alternative means of selecting neutralization-resistant variants.

With this or a comparable panel of monoclonal antibodies having limited neutralizing capabilities, the isolation of neutralization-resistant variants by simply screening for virus that escapes neutralization would be arduous at best because of the large percentage of wild-type (wt) virus surviving treatment. Although NDV exhibits a high mutation rate, with spontaneous mutants sometimes being isolated at frequencies of 1–2% (14), the high persistent fraction of nonneutralized virus with antibody attached to it would obscure resistant variants present at much lower frequencies. We now describe the use of rabbit anti-mouse Ig to eliminate virus particles that remain infectious despite attached antibody and the isolation of 14 variants. Some of these still bind the antibody used to select them yet retain infectivity. This protocol should have general applicability in instances where the selecting antibody is either not neutralizing or only weakly neutralizing.

MATERIALS AND METHODS

Virus. wt virus of the Australia-Victoria strain (1932) of NDV (AV-wt) and antigenic variants of it, were grown in 10-day-old embryonated hen eggs at 37°C from stocks of virus one-egg passage from cloning (15). After the death of the majority of embryos, allantoic fluid was harvested and stored at –70°C. For some experiments, virus was purified from allantoic fluid as described (16, 17). Protein was determined by the method of Lowry *et al.* (18).

Cells. Secondary chicken embryo cells were prepared and maintained in standard medium as described (15, 19). Confluent secondary cultures (24–48 hr after plating) in 60-mm tissue culture dishes were used for plaque titrations.

Hybridomas and Monoclonal Antibodies. Preparation, screening, and cloning of hybridomas; purification of antibodies from ascites fluids (12); and estimation of antibody concentration (13) have been described.

Passaged Virus Stocks. To increase our chances of isolating variants, we used passaged rather than cloned virus as a source of variants. Cloned AV-wt was serially passaged three times on chicken embryo cells and grown as above in embryonated hen eggs to generate a set of passaged virus stocks.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HN, hemagglutinin-neuraminidase glycoprotein; NDV, Newcastle disease virus; wt, wild type; AV-wt, wild-type virus of the Australian-Victoria strain (1932) of NDV; pfu, plaque-forming units.

Neutralization Assays. To determine persistent fractions, virus [$0.5\text{--}1.0 \times 10^7$ plaque-forming units (pfu)/ml] was incubated with an equal volume of ascites fluid diluted to an antibody concentration of $50 \mu\text{g/ml}$ in Hanks' balanced salt solution (HBSS; GIBCO) for 1 hr at 25°C (13). Neutralization was stopped by serial 1:9 dilution in ice-cold HBSS, and 0.2-ml samples were plated in duplicate on 60-mm plates of chicken embryo cells. After adsorption for 45 min, plaque assays were performed as described (15). Antibodies to the nucleocapsid protein served as controls.

Treatment with Rabbit Anti-Mouse Ig. Following neutralization of $0.5\text{--}2.0 \times 10^8$ pfu/ml, assay mixtures were diluted 1:50 in HBS to diminish "quenching" of the rabbit anti-mouse Ig by unbound monoclonal antibody. Samples were then incubated for an additional 1 hr at 25°C after the addition of $25 \mu\text{g}$ of rabbit anti-mouse Ig (Litton Bionetics) per ml, and dilution and plating were done as described above.

Screening for Variants. Plaques surviving treatment with antibody and rabbit anti-mouse Ig were picked into 0.5 ml of HBSS containing 2% calf serum and agitated vigorously. Fifty microliters of virus suspension was added to $70 \mu\text{l}$ of HBSS and $120 \mu\text{l}$ of selecting monoclonal antibody (at $50 \mu\text{g/ml}$). After 1 hr at 25°C , $200 \mu\text{l}$ of this mixture was plated as described above. A control was treated with antibody to the nucleocapsid protein. Virus from plaques that, when so treated, left $>50\%$ persistent fractions were expanded in eggs and screened again for neutralization by selecting antibody.

RIA. Antibody binding to purified virus was determined by solid-phase RIA (12) with intact virions as antigen. Antibody was added in serial 1:2 dilutions in gamma-globulin-free horse serum (GIBCO) from an initial dilution of ascites fluid of 1:100. The reciprocal of the dilution of ascites fluid at the midpoint of the exponential part of a semilogarithmic plot of cpm bound vs. the dilution of ascites fluid is taken as the titer. Relative avidity was estimated by the method of Stone and Nowinski (20), which assumes that, in titration curves of antibody binding to virus in a solid-phase RIA, the maximum amount of antibody bound at saturation equilibrium directly reflects the binding constant (avidity) of the complex.

RESULTS

Selection of Variants. Variants were selected from plaques surviving treatment of passaged stocks with monoclonal antibody and rabbit anti-mouse Ig. Only one variant from each stock was chosen for each antibody unless the variants were clearly different as discerned by their neutralization properties. This reduces the likelihood of characterizing duplicates of the same variant. Treatment with monoclonal antibody and rabbit anti-mouse Ig resulted in a reduction in infectivity of at least 4 orders of magnitude. Surviving plaques were not all derived from variants but yielded variants at widely varying frequencies depending on the selecting antibody (data not shown).

The variants, named for the selecting antibody, are listed in Table 1. We isolated 14 variants, using 7 anti-HN monoclonal antibodies encompassing the four antigenic sites on HN (12). Treatment of any variant with selecting antibody resulted in a $>50\%$ persistent fraction. Given the limitations of the plaque assay and the fact that these data are from a single representative experiment, the comparatively negligible neutralization is not significant.

Identification of Variants that Bind Selecting Antibody Without Significant Neutralization. To determine whether failure to be neutralized by the selecting antibody was due to inability to bind that antibody, we treated the mixture with rabbit anti-mouse Ig to detect antibody on virions. We previously have shown that treatment of AV-wt antibody mixtures with rabbit anti-mouse Ig neutralizes infectivity an additional 3 orders of magnitude (13). For each variant, the

Table 1. Neutralization of variants of AV-wt by the selecting antibody with and without subsequent treatment with rabbit anti-mouse Ig

Variant	Selecting antibody*	Persistent fraction, %	
		Selecting antibody alone†	Selecting antibody with RAM Ig‡
V1 _a -1	HN1 _a (1.5)	82	41
V1 _b -1	HN1 _b (1.7)	65	91
V1 _b -2	HN1 _b	117	57
V1 _b -3	HN1 _b	66	81
V1 _b -4	HN1 _b	154	4.5
V1 _c -1	HN1 _c (1.1)	90	72
V2 _a -1	HN2 _a (5.4)	57	<0.02
V2 _a -2	HN2 _a	80	54
V3 _a -1	HN3 _a (11)	96	89
V3 _a -2	HN3 _a	97	87
V4 _a -2	HN4 _a (19)	61	0.1
V4 _b -2	HN4 _b (20)	88	86
V4 _b -3	HN4 _b	66	80
V4 _b -4	HN4 _b	51	5.6

*Persistent fraction remaining after treatment of AV-wt with antibody (in parentheses).

†Persistent fraction surviving treatment with the antibody used to select the variant.

‡Persistent fraction surviving treatment with the antibody used to select it followed by the addition of rabbit anti-mouse (RAM) Ig.

persistent fraction surviving homologous antibody and subsequent treatment with rabbit anti-mouse Ig is also shown in Table 1. For most variants, the addition of rabbit anti-mouse Ig to monoclonal antibody-treated virus had little or no effect—i.e., the persistent fraction in each case remained $>40\%$. Thus, these are classical variants that do not bind homologous antibody. However, treatment with rabbit anti-mouse Ig of variants V1_b-4, V2_a-1, V4_a-2, and V4_b-4 pre-treated with homologous antibody resulted in significant neutralization—in the case of V2_a-1, below the level of detection. For these variants, the amino acid substitutions evidently changed the configuration of the epitope enough that the selecting antibody did not neutralize but bound and sensitized the virus to neutralization by rabbit anti-mouse Ig. Interestingly, two variants, V1_b-4 and V4_b-4 had persistent fractions of 5%, even after treatment with rabbit anti-mouse Ig.

RIAs of Antibody Binding to Variants. We have shown previously by the method of Stone and Nowinski (20) that the different properties of the antibodies, designated HN1–HN4, to the four HN sites do not reflect avidity differences (13). A similar series of solid-phase RIA titration curves is shown in Fig. 1 for each of the site 1 antibodies and their variants as well as the antibodies and AV-wt, as controls. Fig. 1A shows the binding of antibody HN1_a to AV-wt and V1_a-1. Relative to AV-wt, the selecting antibody does not recognize the variant as evidenced by its failure to bind to the variant in the RIA. Thus, V1_a-1 is a "classical" variant, not neutralized by the antibody used to select it (Table 1); it does not bind that antibody (Fig. 1A).

Fig. 1B shows similar curves for the binding of antibody HN1_b to AV-wt and the four variants selected with this antibody. Again, variants selected with this antibody are of the classical type; the selecting antibody exhibited negligible binding (Fig. 1B) and did not neutralize (Table 1). The binding of antibodies HN1_a (Fig. 1A), HN1_b (Fig. 1B), and HN1_c (Fig. 1C) to the respective variant selected with them was negligible. Low-level binding of antibody HN1_b to its variants at the highest antibody concentrations reflects the comparatively high titer of this particular ascites preparation. Failure to

FIG. 1. Binding of antibodies HN1_a (A), HN1_b (B), and HN1_c (C) to AV-wt (●) and to variants selected with them: V1_a-1 (○ in A), V1_b-1 (○ in B), V1_b-2 (■), V1_b-3 (▲), V1_b-4 (▲), and V1_c-1 (○ in C). The initial dilution of ascites was 1:100.

bind selecting antibody is not surprising for any of these variants (except V1_b-4). Treatment of these variants with selecting antibody and rabbit anti-mouse Ig gave no neutralization (Table 1). Thus, these are also "classical" variants (i.e., they are not neutralized and do not bind selecting antibody). Failure of V1_b-4 to bind selecting antibody in the RIA despite its neutralization by rabbit anti-mouse Ig (Table 1) may mean that neutralization by the Ig and binding in the RIA measure different phenomena. Rabbit anti-mouse Ig may somehow facilitate or stabilize binding of antibody to this variant.

Variants V2_a-1 and V2_a-2 were selected for resistance to neutralization by antibody HN2_a (Table 1). However, they clearly differ; antibody HN2_a bound to V2_a-1 as well as it did to AV-wt but did not recognize V2_a-2 (Fig. 2). This agrees with the data obtained with rabbit anti-mouse Ig treatment, where V2_a-1 was neutralized and V2_a-2 was not. Thus, V2_a-1 is a variant for which binding of neutralizing antibody is completely independent of actual neutralization.

The two variants selected with antibody HN3_a were both of the classical type, being recognized like AV-wt by the selecting antibody in the RIA (Fig. 3) in agreement with the failure of rabbit anti-mouse Ig to neutralize (Table 1).

Using two site 4 antibodies, we isolated three types of variants. The RIA binding curve for V4_a-2, selected with antibody HN4_a, shows that this variant bound the selecting

FIG. 2. Binding of antibody HN2_a to AV-wt (●) and to variants V2_a-1 (○) and V2_a-2 (■). The initial dilution of ascites was 1:100.

antibody as well as AV-wt did (Fig. 4A), accounting for its great sensitivity to neutralization by selecting antibody and rabbit anti-mouse Ig (Table 1). Thus, this variant, like V2_a-1, completely separates binding of neutralizing antibody from neutralization.

Of the three variants selected with antibody HN4_b, two (V4_b-2 and V4_b-3) were of the classical type; they did not bind selecting antibody in the RIA (Fig. 4B), confirming the failure of rabbit anti-mouse Ig to neutralize these variants pretreated with HN4_b (Table 1). However, V4_b-4 was quite different. It did not bind selecting antibody in the RIA yet was partially neutralized by rabbit anti-mouse Ig (Table 1). Thus, this variant is similar to V1_b-4 described above (Table 1 and Fig. 1B).

DISCUSSION

Neutralization-resistant variants were isolated by using antibodies to four previously identified antigenic sites on the HN of NDV. This was accomplished, despite the high-persistent fractions of virus that retained infectivity, by treating virus/antibody mixtures with rabbit anti-mouse Ig to neutralize "sensitized" virus—that with bound antibody. Although not all plaques surviving treatment with rabbit anti-mouse Ig result from variants, the overall frequency of variant isolation from passaged variant stocks was not unlike (data not shown) the frequency of variants, $10^{-4.5}$ to $10^{-4.7}$, in cloned stocks of Sendai, vesicular stomatitis, and influenza viruses (21).

Antigenic selection of NDV variants has been described by others but not by the protocol we have used. The isolation of

FIG. 3. Binding of antibody HN3_a to AV-wt (●) and to variants V3_a-1 (○) and V3_a-2 (■). The initial dilution of ascites was 1:100.

FIG. 4. Binding of antibodies HN4_a (A) and HN4_b (B) to AV-wt (●) and to variants selected with them: V4_a-2 (○ in A), V4_b-2 (○ in B), V4_b-3 (■), and V4_b-4 (□). The initial dilution of ascites was 1:100.

variants of NDV strain Ulster 2C with a monoclonal antibody to HN required the presence of selecting antibody in the overlay in the plaque assay (22). Isolation of variants from NDV strain D26 required neither rabbit anti-mouse Ig nor the addition of antibody to the overlay (11); variants were isolated by simple antibody treatment of virus as described for other viruses. The difference between our system and those of the other two groups is not clear but is probably not due to a failure on our part to isolate the "right" monoclonal antibody. First, our panel of antibodies is more complete; functional inhibition studies suggest that the monoclonal antibodies selected by the other groups probably identify a subset of the sites we have identified (11, 23–25). Second, mixtures of antibodies to all four antigenic sites we have delineated on HN neutralize to the same level as polyvalent mouse anti-NDV antiserum (13). Moreover, Kingsford (26) has shown similar high-persistent fractions of monoclonal antibody-treated LaCrosse virus. Third, 16 additional anti-HN antibodies to AV-wt, prepared and graciously provided by Mark Peeples, all leave high-persistent fractions of non-neutralized virus (data not shown).

The majority of our variants are classical variants (i.e., their failure to be neutralized reflects inability to bind selecting antibody). However, our protocol has yielded other variants for which antibody binding and neutralization are separate. Among the fourteen variants we have described thus far, four are unique. Variants V1_b-4 and V4_b-4 bind homologous antibody as detected by treatment with rabbit anti-mouse Ig, yet are only minimally neutralized. Their failure to bind antibody in the RIA suggests that this and neutralization by rabbit anti-mouse Ig do not measure the same phenomenon.

Variants V2_a-1 and V4_a-2 represent a third class. Selecting antibodies bind to these variants with avidities similar to their binding to AV-wt (Fig. 2 and 4A), yet they are only neutralized with rabbit anti-mouse Ig treatment (Table 1). Thus, these variants completely separate neutralizing antibody binding from neutralization. Since antibody binding avidity is not affected, we assume that the epitope is intact in the variant. However, the conformation of the variants' HN may

be altered such that the epitope is no longer near a critical site for neutralization. It should be pointed out that these mutants actually may have fortuitously survived the selection process; mutants of this phenotype would be only weakly selected, or perhaps in the extreme example, not even selected at all by rabbit anti-mouse Ig treatment of sensitized virus.

The phenomenon of "sensitized" virus—i.e., infectious virus with bound antibody—is not without precedent. It has been seen with early antisera to NDV (27) and herpes simplex virus (28). More importantly, Ashe and Notkins (29) demonstrated a significant persistent fraction for herpes simplex virus treated with rabbit antiserum. That anti-Ig was able to further neutralize this "sensitized" virus indicated that the persistent fraction contained infectious virus-antibody complexes. It was proposed that the persistent fraction resulted from blocking of so-called "critical sites" for neutralization by nonneutralizing antibodies. That such phenomena exist in polyvalent sera is strongly suggested by the recent demonstration that a nonneutralizing monoclonal antibody to the major glycoprotein of mouse mammary tumor virus blocks neutralization by a second neutralizing antibody (30). This obviously would not be a consideration in a system of viral particles sensitized by a single monoclonal antibody population.

We have eliminated several possible causes of high-persistent fractions: aggregation, a host-cell-specific effect, and insufficient antibody (13). How can a monoclonal antibody bind to all particles, yet neutralize only some? Why does a large fraction of virus treated with any of these monoclonal antibodies retain infectivity despite bound antibody? Answers to these questions will be central to an understanding of the mechanism of neutralization of NDV.

Anti-Ig treatment to facilitate isolation of antigenic variants may have considerable general applicability to other systems in which antibodies are weakly neutralizing or even non-neutralizing. Examples of such systems include sensitizing but nonneutralizing antibodies to the neuraminidase of influenza virus (31) and to mouse mammary tumor virus (30), nonneutralizing antibodies to the E1 glycoprotein that passively protect mice from lethal alphavirus encephalitis (32), antibodies to LaCrosse virus that leave high persistent fractions (26), and antibodies to the F and HN glycoproteins of NDV, which select variants only when incorporated in the agarose overlay of the plaque assay (22). We are presently applying the protocol to the isolation of variants with three monoclonal antibodies to the NDV HN that do not neutralize but sensitize virus to neutralization by anti-Ig.

The authors thank Jody Borgman, Bruce Kahn, Kenneth Lawton, and Peter Nicholson for excellent technical assistance; Nancy Graham for manuscript preparation; and Rhona Glickman, Tracy Morrison, Mark Peeples, Lyn Schmidt, and Rick Syddall for helpful discussions and criticisms. This investigation was supported by a Public Health Service grant from the National Institute of Allergy and Infectious Diseases (AI 12467), an institutional Biomedical Research Support Grant (RR-05712), and a National Multiple Sclerosis Society fellowship to R.M.I.

1. Caton, A. J., Brownlee, G. G., Yewdell, J. W. & Gerhard, W. (1982) *Cell* **31**, 417–427.
2. Gerhard, W. & Webster, R. G. (1978) *J. Exp. Med.* **148**, 383–392.
3. Gerhard, W., Yewdell, J. & Frankel, M. E. (1981) *Nature (London)* **290**, 713–717.
4. Laver, W. G., Air, G. M., Webster, R. G., Gerhard, W., Ward, C. W. & Dopheide, T. A. A. (1979) *Virology* **98**, 226–237.
5. Laver, W. G., Gerhard, R. G., Webster, R. G., Frankel, M. E. & Air, G. M. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1425–1429.
6. Webster, R. G., Laver, W. G., Air, G. M., Ward, C.,

- Gerhard, W. & van Wyke, K. L. (1980) *Ann. N.Y. Acad. Sci.* **354**, 142-162.
7. Lefrancois, L. & Lyles, D. S. (1982) *J. Immunol.* **130**, 394-398.
 8. Wiktor, T. J. & Koprowski, H. (1980) *J. Exp. Med.* **152**, 99-112.
 9. Portner, A. (1981) *Virology* **115**, 375-384.
 10. Yewdell, J. & Gerhard, W. (1982) *J. Immunol.* **128**, 2670-2675.
 11. Nishikawa, K., Isomura, S., Suzuki, S., Watanabe, E., Hamaguchi, M., Yoshida, T. & Nagai, Y. (1983) *Virology* **130**, 318-330.
 12. Iorio, R. M. & Bratt, M. A. (1983) *J. Virol.* **48**, 440-450.
 13. Iorio, R. M. & Bratt, M. A. (1984) *J. Virol.* **51**, 445-451.
 14. Bratt, M. A. & Hightower, L. E. (1977) in *Comprehensive Virology*, eds. Fraenkel-Conrat, H. & Wagner, R. R. (Plenum, New York), Vol. 9, pp. 457-533.
 15. Bratt, M. A. & Gallaher, W. R. (1969) *Proc. Natl. Acad. Sci. USA* **64**, 536-543.
 16. Clavell, L. A. & Bratt, M. A. (1972) *Appl. Microbiol.* **23**, 461-470.
 17. Weiss, S. R. & Bratt, M. A. (1974) *J. Virol.* **13**, 1220-1230.
 18. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Bio. Chem.* **193**, 265-275.
 19. Hightower, L. E. & Bratt, M. A. (1974) *J. Virol.* **13**, 788-800.
 20. Stone, M. R. & Nowinski, R. C. (1980) *Virology* **100**, 370-381.
 21. Portner, A., Webster, R. G. & Bean, W. J. (1980) *Virology* **104**, 235-238.
 22. Russell, P. H. (1984) *J. Gen. Virol.* **65**, 795-798.
 23. Russell, P. H., Griffiths, P. C., Goswami, K. K. A., Alexander, D. J., Cannon, M. J. & Russell, W. C. (1983) *J. Gen. Virol.* **64**, 2069-2072.
 24. Iorio, R. M. & Bratt, M. A. (1984) in *Nonsegmented Negative Strand Viruses*, eds. Bishop, D. H. L. & Compans, R. W. (Academic, New York), pp. 309-314.
 25. Iorio, R. M. & Bratt, M. A. (1984) *J. Immunol.* **133**, 2215-2219.
 26. Kingsford, L. (1984) *Virology* **136**, 265-273.
 27. Linscott, W. D. & Levinson, W. E. (1969) *Proc. Natl. Acad. Sci. USA* **64**, 520-527.
 28. Yoshino, K. & Taniguchi, S. (1965) *Virology* **26**, 44-53.
 29. Ashe, W. K. & Notkins, A. L. (1966) *Proc. Natl. Acad. Sci. USA* **56**, 447-451.
 30. Massey, R. J. & Schochetman, G. (1981) *Science* **213**, 442-449.
 31. Schmaljohn, A. L., Johnson, E. D., Dalrymple, J. M. & Cole, G. A. (1982) *Nature (London)* **297**, 70-72.
 32. Majer, M. & Link, F. (1971) *J. Gen. Virol.* **13**, 355-356.