

Key words: *Junin virus*/antigenic variants/kinetic neutralization assay

Differentiation of Junin Virus and Antigenic Variants Isolated *in vivo* by Kinetic Neutralization Assays

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(Accepted 5 May 1988)

SUMMARY

The major natural reservoir of Junin virus, the aetiological agent of Argentine haemorrhagic fever, is the cricetid *Calomys musculinus*. Neonatal animals experimentally infected with Junin virus (XJCl₃ strain) developed typical disease and approximately 80% of them died. Most survivors become persistently infected. Antigenically variant viruses were isolated from the blood and brain of infected cricetids during the acute and chronic stages of the disease. These variants could be distinguished from the parental strain by kinetic neutralization assays using polyclonal antibodies. Some biological properties were shared with the parental virus strain including its virulence for newborn *C. musculinus*. These variant viruses may play a major role in chronic disease since we have shown that a viral isolate from an infected brain was poorly neutralized by serum obtained from the same animal.

Junin virus (JV), the aetiological agent of Argentine haemorrhagic fever (AHF), is an arenavirus that establishes a natural, persistent infection in the cricetid *Calomys musculinus* (Laguens *et al.*, 1982; Lampuri *et al.*, 1982). In order to determine the mechanism(s) involved in viral persistence, an experimental model with *C. musculinus* infected with the XJCl₃ strain of JV was developed in our laboratory (Laguens *et al.*, 1982; Lampuri *et al.*, 1982).

Previous work showed that newborn animals inoculated by the intraperitoneal (i.p.) route became infected and although approximately 60% of them died, the others recovered (Lampuri *et al.*, 1982). Interestingly, it was possible to detect neutralizing antibodies (NA) in surviving cricetids that had high virus titres in their brains indicating that they were chronically infected (Laguens *et al.*, 1982).

The inefficiency of circulating NA in clearing the virus could be explained by the emergence of antigenic variants of JV, which were detected in brain and blood of persistently infected animals by cross-neutralization assays against an antiserum raised to parental virus (Lampuri & Coto, 1983; Alché & Coto, 1986).

In this paper, we present evidence that kinetic neutralization reactions allowed a clear distinction between parental JV and antigenic variants isolated from infected *C. musculinus*. These assays also revealed that virus obtained from cricetid brain was poorly neutralized by serum from the same animal, providing a clue to the mechanism of *in vivo* viral persistence.

Kinetic neutralization tests were performed in triplicate, mixing equal volumes of inactivated antiserum (at a dilution that neutralized 90% of the homologous virus in 5 min), and viral suspensions containing 200 p.f.u./0.2 ml. Controls were done with fresh guinea-pig serum. Mixtures were incubated for 5, 10 and 15 min at 37 °C and assayed for residual virus infectivity by plaque formation on Vero cell monolayers. Preliminary experiments performed with parental JV showed that viral inactivation occurred as a straight-line relationship at 2 and 5 min of incubation. In order to calculate the *K* value, we chose the 5 min point. Data obtained conform to the equation $K = (D/t)2.3 \log (V_0/V)$, where V_0 and V are the viral concentration in the control mixture and in the reaction mixture at time t respectively, and D is the inverse of the

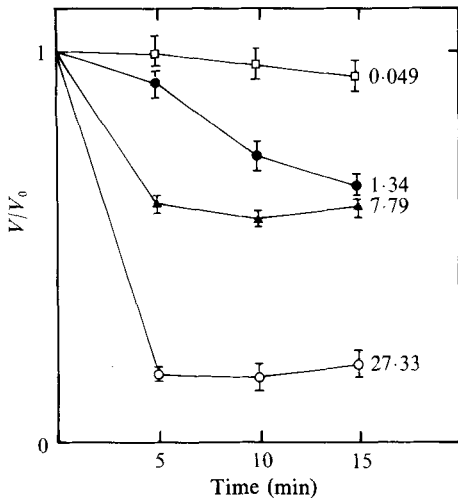


Fig. 1

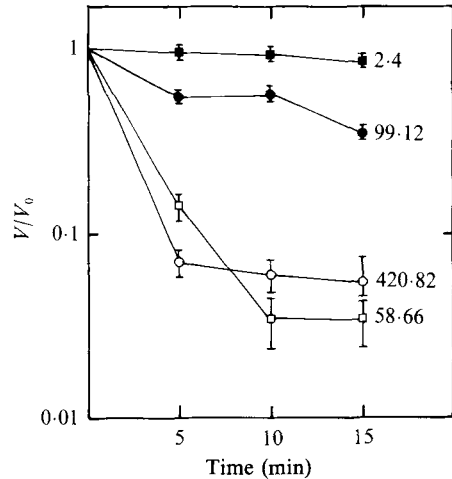


Fig. 2

Fig. 1. Kinetic neutralization curves of JV (○), 192(35) (□), 215(61) (▲) and 117(23) (●) antigenic variants with antiserum prepared to JV (1:160). The numbers by each curve are the K value for the antiserum with the particular virus. V_0 and V are the viral concentration in the control mixture and in the reaction mixture at the times indicated.

Fig. 2. Kinetic neutralization curves of JV_a (□) and virus from *C. musculus* 109 brain (■) with antiserum prepared to JV (1:160) and of JV_a (○) and virus from *C. musculus* 109 brain (●) with serum obtained from *C. musculus* 109 (1:800). The numbers by each curve are the K values for each antiserum with the particular virus. V_0 and V are the viral concentration in the control mixture and in the reaction mixture at the times indicated.

antiserum dilution. K is a measure of serum neutralizing capacity, valid only when obtained from the straight-line portion of the plot of the neutralization reaction under conditions where antibody is present in excess (Waner & Weller, 1978).

Immune serum against parental JV was raised in a guinea-pig (Alché & Coto, 1986), with an NA titre of 320, as determined by a standard neutralization reaction. Kinetic neutralization tests were performed with virus isolates from the blood of *C. musculus* 192, 117 and 215 obtained at 35, 23 and 61 days post-infection (p.i.), respectively (Alché & Coto, 1986).

The results plotted in Fig. 1 show a differential reactivity of anti-JV antibodies with parental virus and variants. As expected, a very rapid decrease of JV infectivity was observed during the first 5 min and, thereafter, the reaction decelerated to attain values corresponding to residual virus infectivity. In contrast, the kinetic neutralization pattern of JV variants was completely different: by 5 min, infectivity values of 99, 92 and 61% were obtained for 192(35), 117(23) and 215(61) viruses, respectively. Therefore, kinetic neutralization tests showed differences among variants as indicated by the corresponding K values, which ranged from 27.33 (parental JV) to 0.049 [192(35) virus].

Since JV variants were unequivocally detected by neutralization kinetics, it was considered of interest to determine whether viral isolates from the brains of infected cricetids could still be neutralized by sera from the same animals.

To investigate this, 13 (2- to 4-day-old) *C. musculus* were inoculated with 4×10^3 LD₅₀ of JV i.p. and sacrificed between 13 and 116 days p.i. The detection of virus in brain was performed as described (Laguens *et al.*, 1982); serum NA titres were determined in cross-neutralization tests against parental JV grown in cricetid brain (JV_a).

The results in Table 1 indicate that all *C. musculus* studied mounted a humoral response from day 18 p.i. onwards, but only some became persistently infected. The simultaneous presence of infectious virus in brain and serum NA was detected only in *C. musculus* 109, 117 and 203. For

Table 1. Isolation of JV from cricetid brain and detection of NA

<i>C. musculus</i> number	Day of sacrifice (p.i.)	Brain viral titre (p.f.u./ml)	NA titre*
101 A	13	ND†	20
107	14	3.12×10^5	<10
101 B	15	<5	<10
102	16	3×10^3	ND
108	18	ND	120
109	20	2.8×10^6	1600
117	23	3.5×10^3	120
118	24	<5	ND
110 A	26	1.95×10^6	ND
110 B	27	<5	1600
99	64	7.25×10^4	ND
203	112	3×10^3	800
305	116	<5	800

* Values represent reciprocals of the highest dilutions of sera obtained from infected *C. musculus* that neutralized 80% of plaque formation by JV_b.

† ND, Not done.

further experiments, we selected *C. musculus* 109 because it exhibited both high virus and NA titres. Initially, we wanted to determine whether *C. musculus* 109 virus behaved as an antigenic variant of JV. For that purpose, a kinetic neutralization test was done with an anti-JV serum (1:160). The kinetic neutralization pattern of *C. musculus* 109 virus ($K = 2.4$) differed significantly from that of parental JV ($K = 58.66$) (Fig. 2), and so the former can be considered an antigenic variant.

In parallel, we compared the neutralization reactivity of *C. musculus* 109 serum when assayed against *C. musculus* 109 brain virus and JV_b. As can be seen in Fig. 2, *C. musculus* 109 serum reacted differentially with JV_b and its homologous virus. The corresponding K values indicate that *C. musculus* 109 antibodies had a fourfold increased reactivity for parental JV_b in comparison with its own virus. Thus, serum from infected *C. musculus* poorly neutralized virus isolated from the same animal, whereas it still recognized the virus that caused infection.

These results suggest that an alteration must have occurred in the antigens of the viral variants to which antibodies are directed. A similar situation has been observed in the case of visna virus; antiserum obtained early in infection from a sheep inoculated with the viral strain 1514 distinguished 1514 virus from viral isolates LV₁₋₁ and LV₁₋₄ obtained during the course of persistent infection (Narayan *et al.*, 1978; Scott *et al.*, 1979). Likewise, equine infectious anaemia virus (EIAV) isolates could be differentiated antigenically by neutralization assays with serum taken from the host animal at various times between disease episodes (Salinovich *et al.*, 1986).

The distinctive neutralization behaviour of JV, 192(35) and 215(61) viruses (Fig. 1) did not correlate with changes in biological properties studied *in vitro*. Thus, it was not possible to detect differences in their growth curves at 37 °C. The efficiency of plating (40 °C/37 °C) was nearly 0.06 in all cases, suggesting that the antigenic alteration was not associated with a temperature-sensitive phenotype that was spontaneously selected after the establishment of a persistent infection with JV *in vitro* (Damonte & Coto, 1979). Likewise, these antigenic variants retained their pathogenicity for neonatal cricetids. They caused typical disease although they showed higher virulence for cricetids than parental JV because deaths occurred earlier, probably due to host adaptation. Thus, when 2- to 4-day-old *C. musculus* were inoculated intraperitoneally with 300 LD₅₀ of JV or variant virus, differences among cumulative percentages of mortality were registered (Fig. 3). In all cases, the first deaths occurred around 10 and 14 days p.i., but thereafter the kinetics of mortality varied depending on the inoculated virus. Maximum values (approx. 90%) were reached between 17 and 21 days p.i. for cricetids inoculated with viral variants [117(23) and 215(61) viruses], whereas a value of 86% was attained at 40 days in the case

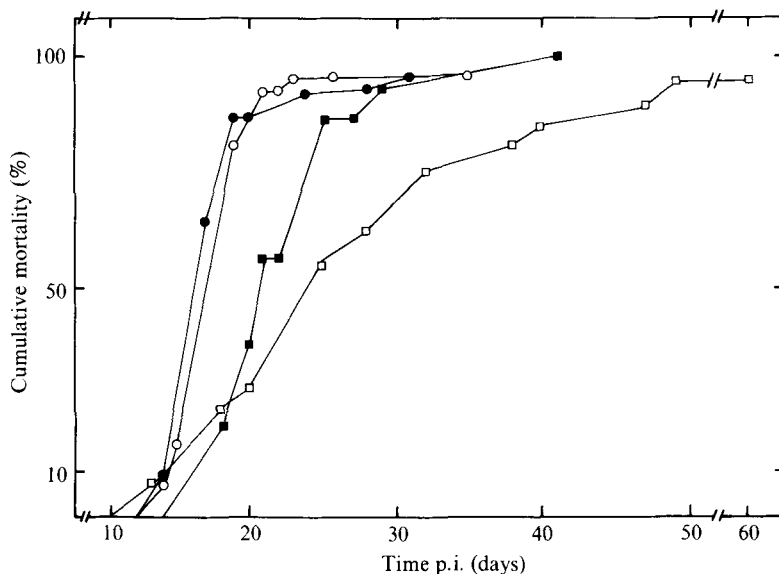


Fig. 3. Cumulative mortality percentages of 2- to 4-day-old *C. musculus* inoculated i.p. with 300 LD₅₀ of JV (□), 192(35) (■), 215(61) (●) and 117(23) (○) viruses. Each curve represents the data from 21, 16, 31 and 27 infected cricetids, respectively.

of *C. musculus* inoculated with JV (Fig. 3). A mortality value of 90% was reached at 29 days p.i. in the cricetids inoculated with 192(35) virus. The Kolmogorov-Smirnov test (Daniel, 1978) revealed significant differences among curves corresponding to JV and the variants at $P = 0.995$, with the exception of 192(35) virus.

As has been observed with several RNA viruses (Steinhauer & Holland, 1987), the results reported here suggest that JV could elude the antiviral immune response and persist in the infected host by the emergence of antigenically new virus progeny. However, it was not possible to determine whether these antigenic variants arose *de novo* during infection or had been present in the parental stock and were selected by growth *in vivo*. Evidence favouring the second possibility is supported by experiments performed *in vitro*, which demonstrated that variants could be selected by culturing parental JV in the presence of NA (data not shown), indicating that the viral stock contained a phenotypically mixed population even after plaque purification.

In nature, antigenic drift would be an additional mechanism to explain viral persistence, since it is possible to detect virus and anti-JV antibodies even in residents of an area not endemic for AHF (Weissenbacher *et al.*, 1985). Considering that naturally infected *C. musculus* may carry JV in their blood (Sabattini *et al.*, 1977) and saliva (Martínez Peralta *et al.*, 1979), variant viruses could well emerge in wild cricetids. Likewise, variants isolated from experimentally infected animals were distinguished by polyclonal antiserum and, hence, would be of epidemiological significance since selection with monoclonal antibodies frequently produces virus with no survival advantage in nature (Webster & Laver, 1980).

We still do not know how many epitopes are involved in the antigenic change; further studies with monoclonal antibodies will help to define the number of relevant sites on the viral glycoprotein of the antigenic variants, as has been demonstrated for Tacaribe virus (Howard *et al.*, 1985).

Therefore, JV, as well as other viruses such as visna virus (Narayan *et al.*, 1978) and EIAV (Salinovich *et al.*, 1986), could be classified as being capable of persistent infection in which replication of the agent can occur in a competent immune environment. The arising antibody response would cause the formation of immune complexes, as was suggested by the inhibition of antibody-dependent cellular cytotoxicity assays performed with sera of JV-infected animals (Coulombié, 1986).

This research was supported by grants from Secretaría de Ciencia y Técnica, Universidad de Buenos Aires. L.E.A. received a Fellowship from the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina (CONICET). C.E.C. received a Research Career award from CONICET.

REFERENCES

- ALCHÉ, L. E. & COTO, C. E. (1986). Antigenic variants of Junín virus isolated from infected *Calomys musculinus*. *Archives of Virology* **90**, 343–348.
- COULOMBIÉ, F. C. (1986). *Immunobiología de Calomys musculinus infectado experimentalmente con virus Junín (agente etiológico de la fiebre hemorrágica argentina)*. Ph.D. thesis, Universidad de Buenos Aires.
- DAMONTE, E. B. & COTO, C. E. (1979). Temperature sensitivity of the arenavirus Junín isolated from persistently infected Vero cells. *Intervirology* **11**, 282–287.
- DANIEL, W. W. (1978). Goodness-of-fit tests. In *Applied Nonparametric Statistics*, pp. 254–289. Boston: Houghton Mifflin.
- HOWARD, C. R., LEWICKI, H., ALLISON, L., SALTER, M. & BUCHMEIER, M. J. (1985). Properties and characterization of monoclonal antibodies to Tacaribe virus. *Journal of General Virology* **66**, 1383–1395.
- LAGUENS, R. M., LAMPURI, J. S., COTO, C. E. & LAGUENS, R. P. (1982). Patología de la infección persistente del *Calomys musculinus* con una cepa atenuada de virus Junín. *Medicina, Buenos Aires* **42**, 273–278.
- LAMPURI, J. S. & COTO, C. E. (1983). Responsabilidad de las variantes serológicas en la infección crónica con virus Junín. *Medicina, Buenos Aires* **43**, 733.
- LAMPURI, J. S., VIDAL, M. C. & COTO, C. E. (1982). Respuesta del *Calomys musculinus* a la infección experimental con virus Junín. *Medicina, Buenos Aires* **42**, 61–66.
- MARTÍNEZ PERALTA, L. A., LAGUENS, R. P., COSSIO, P. M., SABATTINI, M. S., MAIZTEGUI, J. I. & ARANA, R. M. (1979). Presence of viral particles in the salivary gland of *Calomys musculinus* infected with Junín virus by a natural route. *Intervirology* **11**, 111–116.
- NARAYAN, O., GRIFFIN, D. E. & CLEMENTS, J. E. (1978). Virus mutation during 'slow infection': temporal development and characterization of mutants of visna virus recovered from sheep. *Journal of General Virology* **41**, 343–352.
- SABATTINI, M. S., GONZÁLEZ DE RÍOS, L. E., DÍAZ, G. & VEGA, V. R. (1977). Infección natural y experimental de roedores con virus Junín. *Medicina, Buenos Aires* **37**, 149–161.
- SALINOVICH, O., PAYNE, S. L., MONTELARO, R. C., HUSSAIN, K. A., ISSEL, C. J. & SCHNORR, K. L. (1986). Rapid emergence of novel antigenic and genetic variants of equine infectious anemia virus during persistent infection. *Journal of Virology* **57**, 71–80.
- SCOTT, J. V., STOWRING, L. & HAASE, A. T. (1979). Antigenic variation in visna virus. *Cell* **18**, 321–327.
- STEINHAUER, D. A. & HOLLAND, J. J. (1987). Rapid evolution of RNA viruses. *Annual Review of Microbiology* **41**, 409–433.
- WANER, J. L. & WELLER, T. H. (1978). Analysis of antigenic diversity among human cytomegaloviruses by kinetic neutralization tests with high-titered rabbit antisera. *Infection and Immunity* **21**, 151–157.
- WEBSTER, R. G. & LAVER, W. G. (1980). Determination of the number of nonoverlapping antigenic areas on Hong Kong (H3N2) influenza virus hemagglutinin with monoclonal antibodies and the selection of variants with potential epidemiological significance. *Virology* **104**, 139–148.
- WEISSENBACHER, M. C., CALELLO, M. A., CARBALLAL, G., PLANES, N., DE LA VEGA, M. T. & KRAVETZ, F. (1985). Actividad de virus Junín en humanos y roedores de áreas no endémicas de la provincia de Buenos Aires. *Medicina, Buenos Aires* **45**, 263–268.

(Received 6 January 1988)