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HETEROGENEITY AND STABILITY CHARACTERISTICS OF CANDID 1 ATTENUATED STRAIN OF JUNIN VIRUS

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Summary. - Fourteen clones of Candid 1 strain of Junin virus were studied in terms of their attenuation and stability for 9 days-old mice. The cluster analysis of the efficiency of lethal infection, namely the virulence (lethality) indicates (PFU/LD_{50}) of the clones showed that the strain population is heterogeneous. It is constitued by subpopulations with lower and higher virulence as compared to the parent strain. Moreover, one or two passages through mice and *Calomys musculinus,* the main Junin virus reservoir of this strain produced changes of its virulence meanwhile the *in vitro* passages did not affect its attenuation level.

Key words: Junin virus; vaccine strain; clone selection; population heterogeneity and stability

Introduction

Junin virus (Arenaviridae) causes Argentine Haemorrhagic Fever (AHF) an endemoepidemic, acute and severe disease with a case-fatality rate of 15-30 % (Maiztegui *et al.*, 1979). Inmediately after the isolation of Junin virus in 1958 (Parodi *et al.*, 1958), several attemps have been made to develop an effective vaccine against AHF. What concerns the attenuated vaccine virus, the first one was the XJCL3 strain obtained by cloning the XJ prototype strain in MA-111 heteroploid cells, and further passaged in brain of mice. It was not lethal for adult guinea pigs and confered immunity with high titers of neutralizing antibodies (Guerrero *et al.*, 1969). This step led to the trials in human volunteers under the assumption that the low virulence of XJCL3 strain for guinea pigs implied attenuation for man (Weissenbacher *et al.*, 1969; Ruggiero *et al.*, 1974).

Because of the fact that the strain has been cloned in heteroploid cells and the vaccine was prepared in mouse brain its administration to human volunteers was discontinued.

Furthermore, a quantitative differential virulence of XJCL3 and the wild

strains (CbaFHA5069 and CbaIV4454) for mice and guinea pigs has also been reported (Contigiani and Sabattini, 1984).

Since 1980 attemps were made to replicate and clone the virus using other substrates, more suitable for human vaccine. In this regard, alternative attenuated strains XJO and Candid 1 were obtained from the same parental strain as XJCL3 (Guerrero *et al.*, 1980; Barrera Oro *et al.*, 1985). The advantage of these strains was not having been passaged in heteroploid cells. Candid 1 strain has been developed as potential vaccine by cloning and replication in FRhL-2 cells. It has been shown to be free of adventious agents, attenuated and immunogenic for guinea pigs and non-human primates (Barrera Oro *et al.*, 1985; Lupton *et al.*, 1984). Recently, Dr. J. I. Maiztegui (personal communication) has demonstrated the innocuity, immunogenicity and protective efficacy of this strain in human volunteers.

Although, the attenuation of Candid I strain for animal models was demonstrated, proving its least neurovirulence, neuroinvasiveness and persistence of the strains studied (Medeot *et al.*, 1990; Contigiani *et al.*, 1991), further studies are necessary to determine the critical differences between wild and attenuated strains, Junin virus human vaccine candidates.

The purpose of this work was to study the Candid 1 vaccine strain in terms of its heterogeneity and stability. This became important in the design and testing of live attenuated vaccines of Junin virus.

Materials and Methods

Virus strains. Candid 1 strain, human vaccine (Barrera Oro *et al.*, 1982) was kindly provided by Dr. J. I. Maiztegui, National Institute of Viral Haemorrhagic Disease, Pergamino, Argentina, as the culture supernatant harvested 96 hr p.i. on foetal rhesus lung (FRhL) cells. This strain underwent 2 passages in guinea pigs, 44 in mice and 19 in FRhL cell cultures. To evaluate the possible changes in the virulence of Candid 1 vaccine strain, virus stock passages in different hosts *in vivo* (suckling albino mouse brain and suckling *Calomys musculinus* brain) and *in vitro* (FRhL and Vero cells) were performed.

Animals. Albino mice, *Calomys musculinus* (Cm), main Junin virus reservoir, and guinea pigs outbred in our laboratory were used in this study. In accord with previous works (Contigiani and Sabattini, 1983; Medeot *et al.*, 1990) we selected 9–11 days-old mice, 8–12 months-old Cm and 11 days-old guinea pigs to examine the stability and heterogeneity of Candid 1 vaccine strain.

Cell culture. The cloned 76 Vero cell cultures were employed. Cells were grown in minimal essential Eagle's medium (MEM) with Earle's salt and glutamine supplemented with 10 % foetal calf serum (FCS) and gentamicine (40 μg/ml). One-two day old confluent monolayers were used for virus assays.

Animal studies. Serial tenfold dilutions of Candid 1 strain, each *in vivo* or *in vitro* passage or plaque of this strain, were inoculated in parallel in mice or guinea pigs and Vero cells (PFU determination). Each animal received 0.05 ml by intracerebral route and was observed for clinical signs of illness and death over 21 days for mice and 30 days for guinea pigs. The LD_{50} was calculated in a standard way and the virulence (lethality) index was determined as the log (PFU/LD_{50}) (Contigiani and Sabattini, 1977).

The low availability of Cm of defined age was an impediment to determining the virulence in this host. Instead, lethality rates were obtained from the daily number of deaths ocurring over 60-days period in Cm inoculated with a single dose (3.2 log PFU) of each virus stock.

Plaque selection. Vero cells monolayers in 25 cm^2 flasks were employed for the cloning procedure. Cultures were inoculated with 0.2 ml of terminal dilutions and after 5 days of incubation under nutrient agarose, the monolayers were stained to visualize the plaques. Well isolated plaques were picked by removing the agarose plug from above the plaque and were suspended in MEM with 5 % FCS and gentamicine (Contigiani and Sabattini, 1987). Each plaque was inoculated in the brain of suckling mice for virus propagation. The parent Candid 1 strain were similarly passaged once in Vero cell cultures and once in suckling mice brain. The stocks of each clone and the parent strain were prepared as 10 % suckling mouse brain suspensions in MEM with FCS, clarified at 10 000 rpm and then stored at -80 °C. Differences between the lethality index of the different clones were evaluated by the use of the cluster analysis method that has already been used to compare Influenza virus strains (Weijers *et al.*, 1985; Beyer and Mansurel, 1985; Hartigan, 1975). The Euclidean distance between the lethality values was used to measure the relatedness of the clones. Consequently a distance value of zero would mean identity of the two clones with respect to the lethality index. Thus, clusters of related clones could be defined as based on high similarity.

Table 1. The virulence (lethality) of Candid 1 parent strain and the clones for mice

Cluster	Clone No.	$\log(PFU/LD_{50})$	Statistical values			
			Minimum	Maximum	Mean	SD
I	1	1.400	1.300	1.480	1.405	0.068
	16	1.420				
	18	1.410				
	12	1.420				
	29	1.300				
	11	1.480				
	30	1.330				
II	Candid 1 (Parental strain)	1.720	1.720	1.720	1.720	0.000
III	40	2.770	2.770	3.230	2.900	0.257
	28	2.800				
	74	2.800				
	60	3.230				
IV	7	2.050	2.050	2.390	2.190	0.177
	22	2.130				
	51	2.390				

SD: standard deviation

Results

Heterogeneity of the attenuated strain population

The values of the virulence (lethality) index are shown in Table 1. The fourteen clones tested showed differences in the efficiency of lethality. There could be established 4 different clusters of clones of Candid 1 vaccine strain. The P value < 0.05 obtained (0.001) between the mean lethality values of each cluster confirmed that they are significantly different.

Candid 1 vaccine strain stability

Reversed studies conducted *in vitro* and *in vivo* showed that the attenuation was retained through the *in vitro* passages (Table 2). After the third additional passage through FRhL cells (vaccine substrate) the virulence (lethality) index did not change. However, only one passage through *in vivo* systems produced changes in the lethality index for mice and guinea pigs as compared to the parent strain, meanwhile two passages through suckling albino mouse brain also modified the lethality percent for Cm.

Table 2. The virulence (lethality) of Candid 1 vaccine strain: effect of the passages through in vivo and in vitro systems

Passage level	log(PFU/LD_{50})			No. of dead/total (%)
	9 days-old mice	11 days-old mice	11 days-old guinea pigs	8–12 months-old Cm
Candid 1 (parent strain)	3.10	4.40	4.65	0/24 (0)
Candid 1 + 1 Am	1.72	2.76	1.82	0/19 (0)
Candid 1 + 2 Am	ND	2.37	ND	3/12 (25)
Candid 1 + 1 Am + 1 FRhL	ND	3.46	ND	ND
Candid 1 + 1 Am + 1 Vero	ND	3.46	ND	ND
Candid 1 + 1 Cm	ND	2.54	ND	ND
Candid 1 + 1 Vero	ND	4.17	ND	ND
Candid 1 + 3 FRhL	ND	3.84	ND	ND
Candid 1 + 1 Vero + 1 Am	1.68	3.15	ND	ND

Cm: *Calomys musculinus;* Am: albino mouse; FRhL: foetal rhesus lung cells; ND: not done

Discussion

The present results show that Candid 1 vaccine strain population is as heterogeneous as XJCL3 (Contigiani and Sabattini, 1987), with subpopulations of lower or higher attenuation than the parent strain, at least for the animal model used in these assays. Thus, within the limits of the small number of clones tested, this heterogeneity showed us the necessity to study the stability of the potential vaccine strain. Moreover, through one or two *in vivo* passages Candid 1 strain showed changes in the virulence (lethality) index for the three biological markers (Contigiani and Sabattini, 1977; Contigiani and Sabattini, 1984) studied, while the *in vitro* passages did not affect its attenuation level.

It is well known that the properties of a viral population obtained under given experimental conditions depend not only on the genetic information of the parental strain, but also on the host features favouring to certain virus mutant. The unstability of Candid 1 strain suggests us that either a selection of a viral subpopulation or virus genetic changes ocurred during the *in vivo* passages, changing the potential strain pathogenicity. Moreover, the observed heterogeneity may be due to intrinsic modifications; it is reported that RNA virus genomes are highly unstable (Holland *e al.*, 1982; Vezza *et al.*, 1978).

The observed population heterogeneity and unstability of Candid 1 vaccine strain became important in the design and testing of live attenuated vaccines of Junin virus. Further studies on genome sequence homologies are currently evaluated in order to provide more information on the molecular basis of Junin virus variation.

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