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JUNIN VIRUS REPLICATION IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF PATIENTS WITH ARGENTINE HAEMORRHAGIC FEVER

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Summary. — To study the relationship of Junin virus (JV) to populations of peripheral blood mononuclear cells (PBMC) from patients with Argentine Haemorrhagic Fever (AHF), blood samples were obtained during the acute period of disease and cultured as total, adherent, and non-adherent cell populations. JV was sequentially sought in these cell populations by using an Infectious Centre (IC) assay, whereas free JV in the supernatants was evaluated by plaque formation. IC were obtained in cultures of total PBMC from 8 out of 19 patients. Maximum numbers of IC showed high variation among patients, ranging from 3 to 410 IC per 10^6 viable PBMC. In contrast, IC were sporadically demonstrated in the non-adherent cell population. The release of JV into culture supernatants was detected only in total PBMC cultures, thus in the presence of macrophages. These results demonstrate that circulating monocytes (macrophages) are targets for JV replication contributing to the viral spread in the acute phase of AHF.

Key words: *Arenavirus*; *Junin virus*; *Argentine haemorrhagic fever*; *infectious centre assay*; *human peripheral monocytes* (*macrophages*)

Introduction

Argentine Haemorrhagic Fever (AHF) is an acute systemic disease whic is endemo-epidemic in a wide rural area of Argentina (Maiztegui *et al.*, 1986 Several studies have shown the lymphotropism of Junin virus (JV), th aetiological agent of the disease. The presence of viral antigens associate with changes in lympho-haemopoietic tissues has been so far the most pe manent and specific alteration found in JV infection of humans and lal oratory animals (Maiztegui, 1975; Weissenbacher *et al.*, 1987). These change mainly consisting of bone marrow inhibition or destruction, and lymphocy depletion in lymph nodes (Ponzinibio *et al.*, 1977; González *et al.*, 1980; Ca ballal *et al.*, 1981), have been interpreted as a direct or indirect viral actio leading to immunodepression observed during the acute period of AHF.

Recent studies which demonstrate that JV can be consistently isolated by cocultivation of peripheral blood mononuclear cells (PBMC) of patients with AHF suggest that some of these cells could be infected with JV (Ambrosio *et al.*, 1986). The present work aimed to elucidate the relationship of JV with populations of PBMC from patients with AHF. Studies were performed on cultured total PBMC, as well as adherent and non-adherent cells, obtained on admission from patients with a clinical diagnosis of AHF.

Materials and Methods

Patients. Blood samples were collected on admission (4 to 12 days after the onset of symptoms) from 19 patients with a clinical diagnosis of AHF.

The aetiological diagnosis was established in every case by a seroconversion in neutralization tests (Webb *et al.*, 1969).

Blood mononuclear cells separation and cultivation. PBMC were obtained by centrifugation on Ficoll-Hypaque gradients (Boyum, 1968). The cells at the interface were collected on phosphate-buffered saline (PBS). PBMC were washed twice with, and resuspended in, a medium consisting of RPMI-1640, with 10% inactivated foetal bovine serum (IFBS), antibiotics, and extra-L-glutamine (Complete medium). The cell populations obtained appeared over 95% viable (trypan blue stain), and the homogeneity, monitored by Giemsa stain, was more than 95%.

Half of the total PBMC from each patient was kept in a siliconized glass tube as a cell suspension in Complete medium, containing 2×10^6 cells/ml. Cultures were maintained at 37 °C, in 4% CO_2 for 6 to 10 days.

The remaining half of the PBMC suspension was used to obtain separated adherent and non-adherent cell populations of follows. The cell suspension was adjusted to $5-7 \times 10^6$ cells/ml with Complete medium containing 50% IFBS, placed in plastic tissue culture flasks, and incubated for 2 hr (37 °C, 4% CO_2). This whole procedure was repeated once to allow remaining suspended monocytes to adhere to a fresh plastic surface. Adherent cells were removed from culture flasks by using RPMI-1640 medium containing 2 μg/ml lidocaine, and subcultured in Lab-Tek chambers (Miles Sc. # 4280). These cultures were maintained with Complete medium plus 20% IFBS, and used as macrophage fraction.

Non-adherent cells were collected and maintained as cell suspensions containing 10^6 cell/ml in Complete medium. These cells were used as lymphocyte fraction. Absence of macrophages from the non-adherent cell suspensions was monitored by the addition of immunobeads (Bio-Rad, # 180-1001) into a sample from each culture, and the search of phagocytic cells.

Since marked leukopaenia is one of the prominent features of AHF very low numbers of blood cells were generally obtained; therefore, in 3 of the patients only total PBMC population was examined.

Sampling of cultured cell suspensions. On different days in culture (from 1 to 10) three aliquots were withdrawn from total PBMC and lymphocyte suspensions. The cells were pelleted by centrifugation (250 g, 10 min), and individual supernatants were stored at −70 °C until tested for the presence of JV.

JV detection assays. Intracellular JV was evaluated in total PBMC and lymphocytes by an Infecticus Centre (IC) assay (Ahmed *et al.*, 1987) modified as follows: the cellular pellet from each sample was resuspended as 10^6 cell/ml in RPMI-1640 medium, with 10% heat inactivated human anti-JV serum (NT titre = 10 240). Cells were incubated in a water bath at 37 °C for 1 hr, with gentle mixing every 15 min. Following incubation, the cells were washed three times with the Complete medium. The supernatant of the third washing, performed in a total volume of 1 ml, was saved and inoculated onto Vero cells to score free, non-neutralized JV as PFU.

The washed cell suspension, with a known viable cell concentration (trypan blue stain), was placed undiluted and in five-fold dilutions onto Vero cell monolayers grown in 24 well tissue plates (0.1 ml/well). The plated mononuclear cells were immediately immobilized by adding 0.1 ml of 0.5% agarose in an overlay medium described elsewhere (Webb *et al.*, 1969). After adsorption at 37 °C for 1 hour, 1 ml of the same overlay was carefully added into each well.

Plates were incubated for 4 days at 37 °C in 4% CO_2. Development of plaques was revealed by adding 1 ml/well of a second overlay containing neutral red (0.041 mg/ml) on day 5, incubation for an extra day, then counting plaques on day 6. IC results were expressed per 10^6 viable PBMC inoculated.

In cell-free supernatants JV was titrated by inoculation of undiluted and 10-fold dilutions onto Vero cells grown in 24-well tissue culture plates. The presence of JV was detected by the development of PFU, following procedures described by Webb *et al.* (1969).

Interferon assay. Interferon (IFN) was searched for in the supernatants of total PBMC from 8 patients. On different days in culture, from 1 to 6, samples of supernatants were withdrawn and clarified of cells as described. IFN determinations were performed by inhibition of CPE of vesicular stomatitis virus on Wish cell monolayers (Stewart, 1969). Endogenous α-IFN titres were also evaluated in the serum of each patient studied (Levis *et al.*, 1984).

Results

In 8 out of 19 patients IC assay demonstrated intracellular JV (Table 1). The maximum number of infectious cells obtained in the sequential assays ranged from 3 to 410 per 10^6 viable mononuclear cells. IC from patient 8 (Table 1) rendered uncountable, presumably due to the exceptionally high titre of JV. In this patient, when testing the supernatant of the third washing after treatment of the cells with anti-JV serum, development of PFU showed that non-neutralized JV was still present causing IC to appear confluent.

Sequential IC positive assays from the same patients demonstrated that the numbers of IC tended to increase with the time which PBMC were kept in culture (data not shown). IC were not detected before three days in culture with the exception of severely ill individuals (i.e. patients number 4 and 6 in Table 1, who died of the disease, rendered 33 and 30 IC per 10^6 cells on days 2 and 3 in culture, respectively). Peak titre of JV released into the culture supernatants ranged from undetectable to 7×10^6 PFU/ml.

Table 1. Maximum numbers of IC and JV titres in supernatants of PBMC and lymphocytes from patients with AHF

Patient number	Total PBMC		Lymphocytes	
	IC*	JV titres in supernatants (PFU/ml)	IC	JV titres in supernatants (PFU/ml)
1	163	1×10^2	3	NEG
2	3	NEG	0	NEG
3	10	NEG	3	NEG
4	33	NEG	0	NEG
5	70	6×10^2	ND	ND
6	410	0.4×10^2	10	NEG
7	20	0.4×10^2	0	NEG
8	UNC	7×10^6	ND	ND

* IC per 10^6 viable mononuclear cells plated
UNC — Uncountable; IC appeared confluent

Table 2. Frequency of positive IC assays in blood samples from patients with AHF

Cell population	Time from disease onset (days)			Total (%)
	4 to 6	7 to 9	10 to 12	
Total PBMC	1/10*	5/7	2/2	8/19 (42)
Lymphocytes	1/9	2/6	0/1	3/16 (18.7)

* Positive/total of patients studied

(Table 1). JV was not detected in supernatants from PBMC cultures that rendered negative IC assays.

JV was demonstrated in the supernatant of only two subcultures of macrophages that could be maintained more than 72 hours as confluent monolayers. Maximum titres of 10^2 PFU/ml were obtained in samples of these supernatants collected on day 6 in culture. Table 2 shows that the frequency of IC detection increased by days which elapsed between the clinical onset of the disease and the moment in which blood samples were obtained from the patients.

No IFN activity was detected in the supernatants of total PBMC cultures from 8 patients that were studied for this purpose. Only 3 of 8 cultures (from patients 5, 7, and 8 in Table 1) rendered positive IC assays. In the sera of these three patients the endogenous α-IFN titres were 4, 1280, and 32 IU/ml, respectively. The sera of the remaining five patients had α-IFN titres ranging from 16 to 16 000 IU/ml.

The decay of cell viability, as determined by sequential trypan blue assays, did not show differences that could be attributed to the cytocidal effect of JV.

Discussion

The results reported herein demonstrate that there are productively infected cells among PBMC from the acute period of AHF as shown by their ability to form IC on Vero cell monolayers. Though numbers of IC were generally low and displayed high variations from individual to individual, they were higher in the total PBMC than in the lymphocyte fraction (Table 1). The release of JV into the supernatants, only detected when adherent cells (macrophages) were present in the cultures, indicates that, at a given time, adherent cells carry growing JV.

The low numbers of IC found in PBMC cultures are only a partial representation of the viral involvement of the entire haemopoietic system of AHF patients (González *et al.*, 1980). Thus, the IC detected in blood samples results from infected cells entering the blood stream from haemopoietic tissues. In addition, the virus carried by PBMC could be at different stages of maturation, because: a) in the cell forming IC the intracellularly located virus, would escape neutralization by anti-JV serum of high specific activity; b) the detection of IC improves with the time which PBMC are

kept in culture, thus indicating that an interval is required for intracellular JV to appear as infectious virus; c) unpublished data from our laboratory indicate that JV antigens can be faintly detected by immunofluorescence on acetone-fixed or living PBMC, but only after 5 days in culture.

The increased frequency of IC detection when 7 or more days have elapsed since the clinical onset of AHF (Table 2), can be interpreted as indicative of the extent of haemopoietic tissues involvement in JV infection. The undetectable α-IFN activity in the supernatants of patients' PBMC cultures is consistent with the presence of very low numbers of JV carrying cells. Concomitantly, the finding of high titres of α-IFN in the sera of patients rendering negative IC assays is another evidence suggestive of a larger population of JV-infected lymphoid cells, which cannot be estimated from the peripheral blood cells alone.

The few IC which occasionally appear among non-adherent cells could represent monocyte contaminations, although previous findings would allow to regard them as an underestimation of infected lymphocytes. Studies on peripheral blood cells of JV infected guinea pigs described some morphological alteration in lymphocytes (Carballal *et al.*, 1981*a*), as well as diminished numbers of T cells (Carballal *et al.*, 1981*b*). In patients with AHF, a decrease in T and B lymphocyte populations (Arana *et al.*, 1977) and a transient inversion of T_4/T_8 ratio have been described during the acute period of the disease (Vallejos *et al.*, 1989).

Infectious cells are apparently cleared from the blood of the patients by some mechanism(s) not yet elucidated. On day 4 after the treatment with immune plasma, JV cannot be isolated from the patients' PBMC (Ambrosio *et al.*, 1986), and concomitantly, titres of endogenous α-IFN drop to normal levels (Levis *et al.*, 1984). The low numbers of infectious cells make difficult to rule out the possibility of cytocidal JV infection of PBMC.

The results reported herein demonstrate that in patients with AHF peripheral blood monocytes (macrophages) carry intra-cellular JV, which can replicate in these cells. Circulating monocytes contribute to the spreading of JV different tissues. Viral infection of macrophages can interfere with the role of these cells in the immune response of the patients, thus partially explaining the immunodepression that characterizes the acute period of AHF. Furthermore, if JV multiplication in macrophages is ended by any cytocidal mechanism, subsequent release of reactive products would contribute to a chain of alterations (Peters, 1984). The results of the present study in patients with AHF are consistent with the central role assigned to macrophages in the pathogenesis of arenavirus infections.

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