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IN VIVO JUNIN VIRUS-MOUSE MACROPHAGES INTERACTION

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Summary. — The role of mononuclear phagocytic cells in extraneural infection of the mouse with Junin virus (JV) was studied. Endpoint susceptibility (4 days of life) was evaluated by intraperitoneal (i.p.) inoculation of suckling mice. By means of immunofluorescence (IF) and C3 receptor assays, it was found that macrophages were permissive to viral replication *in vivo* and fostered the recruitment of inflammatory cells as evidenced by the absence of C3 marker. In support, *in vitro* infection failed to induce alterations of this receptor. Throughout, both *in vivo* and *in vitro*, there were no signs of C3-mediated phagocytosis. Silica treatment had no effect on either resistance or susceptibility, suggesting that the "macrophage-barrier" failed to hinder or favour the course of disease. Differences with other JV models are discussed.

Key words: Junin virus; macrophages; silica blockade; immunofluorescence; C3 receptor; mouse

Introduction

In the mouse model, susceptibility to Junin virus (JV) infection depends markedly on both the route of infection and the age of the host. Thus, up to 10 days of age, the suckling mouse presents almost 100 % mortality when infected by intracerebral (i.c.) route. However, these animals prove resistant by intraperitoneal (i.p.) route while still susceptible to i.c. inoculation (Boxaca *et al.*, 1973b). In either case, a common neurological picture involving central nervous system alterations is invariably present in non-survivors, recalling findings in the most severe forms of human disease (Boxaca *et al.*, 1973a).

Probably, age-dependent resistance could involve the appearance of a protective barrier against lethal action. In support, peritoneal macrophages play a major role in many viral infections and, it has been suggested that macrophages mature within the first weeks of life (Mogensen 1979, 1984). In this regard, the mechanisms more widely advanced are macrophage capture with subsequent destruction of virus, or else abortive infection (Mogensen, 1979). Another possibility is replication inhibition of virus-harbou-

ring cells by means of soluble products released by macrophages (Stohlman *et al.*, 1982; Wildy *et al.*, 1982).

In association with JV, macrophages have been recently shown involved in the age-dependent resistance of the cricetid *Calomys musculinus*, a natural JV reservoir (Coulombié *et al.*, 1986). In the rat model, macrophages participate in the course of disease together with the JV strain-dependent effect (Lerman *et al.*, 1986; Blejer *et al.*, 1986). Owing to remarkably early resistance to JV acquired by i.p.-inoculated mouse, the study of macrophage behaviour is essential to understand factors involved in resistance to viral infection.

Accordingly, we have attempted to determine precisely the endpoint of susceptibility, as well as the influence of macrophage blockade and virus--macrophage interaction on the establishment of resistance.

Materials and Methods

Animals. BALB/c suckling and 45-day-old mice raised in our own facilities were used. Litter births dates were strictly controlled twice daily by inspection.

Virus. Prototype XJ and XJCL3 guinea pig-attenuated strains of JV were employed. Stocks were prepared from suckling mouse brain as clarified 10 % (w/v) homogenates in Hanks' solution. Titres as determined by i.c. inoculation in suckling mice were 10^7 LD_{50}/ml in both cases. Plaque forming units (PFU/ml), determined in Vero cells under methylcellulose as described by Giovanniello *et al.* (1981), were found to be 1.23×10^6 and 4×10^6, respectively. Throughout, 10^3 LD_{50} were inoculated by means of suitable dilutions in Hanks solution plus 2 % newborn calf serum (GIBCO) and antibiotics (penicillin-streptomycin).

Susceptibility to i.p. infection. Batches of 1, 2, 3, 4, 5 and 6-day-old mice received 10^3 LD_{50} of either strain in a 0.02 ml vol. Mortality and clinical signs of disease were recorded daily during 30 days post-inoculation (p.i.).

Peritoneal cells (PCs). PC were harvested from ether-killed suckling mice by washing twice the peritoneal cavity with 0.2—0.6 ml of RPMI 1640 plus 10 IU heparin/ml and antibiotics to a final pH 7.2, and from adult animals likewise but using 3 ml.

Cells from each animal were seeded separately in Leighton tubes with glass slides, for 1 hr at 37 °C in a 5 % CO_2 humidified atmosphere, when non-adherent cells were removed by repeated washings with culture medium.

Silica (Si). Silicon dioxide (particle size distribution 0.5—10 μm, 80 % between 1—5 μm, Sigma Chemical Co.) was freed from surface contaminants by repeated boiling in 1 N HCl and then rinsed, following the method described by Le Blanc and Russell (1981), in order to lower toxicity. Si was then sterilized at 180 °C, suspended in saline and sonicated just prior to administration. Suckling mice received 5 mg by i.p. route, while adult mice received 50 mg i.p. or 3 mg intravenously (i.v.). Throughout, virus inoculation took place by the same route as Si, 2 hr later.

Si toxicity for macrophages was assayed *in vitro* as described by Allison *et al.* (1966). *In vivo* toxicity was confirmed on PC removed 1, 2, 3, or 4 days post Si treatment by acridine orange staining (Goldstein and Blomgren, 1973).

Immunofluorescence assay was performed on adherent PCs, by direct standard technique with a fluorescein-labelled human gammaglobulin fraction from a convalescent argentine haemorrhagic fever (AHF) patient. Before staining, cells were fixed in cold acetone for 10 min. As positive controls, BHK_{21} cells infected with XJ strain (multiplicity of infection (MOI): 0.1) were employed, while uninfected BHK_{21} cells and adult macrophages served as negative controls. Slides were observed by epi-ilumination microscopy (Zeiss). Micrographs were taken with Kodak Ektachrome 400 ASA film.

C3 receptor assay was carried out as described by Bianco and Pytowski (1981) using rabbit serum from animals immunized with boiled sheep red blood cell (SRBC) stroma. The 19S fraction was obtained by ammonium sulphate precipitation, and double Sephadex G-200 filtration. The absence of 7S antibodies was confirmed by immunoelectrophoresis. Since the agglutination titre against 0.25 % SRBC was 1/32678, the 19S fraction was employed at 1/1600 dilution against 2.5 % SRBC.

Fresh whole mouse serum diluted 1/5 was used as complement source to render mostly C3b rossettes. In every case, SRBC were used within 15 days after collecting by jugular puncture, and storing in Alserver's solution at 4 °C. No agglutination, ingestion or rossetting was observed in controls in the absence of mouse serum treatment.

Two hundred adherent PCs were counted in each slide, considering those with 3 or more surface SRBC as rossettes. After counting, slides were subjected to hypotonic treatment with Hanks' diluted 1/5 for 15 s, then tonicity restored and phagocytosis was searched for. Micrographs were taken with Agfapan 100 ASA film.

"In vitro" macrophage infection. Resident macrophages from mice 1, 4 or 45 days of life were cultured for 24 hr in RPMI 1640 plus 10 % foetal calf serum (GIBCO), glutamine and antibiotics at 37 °C in a 5 % CO_2 humidified atmosphere, then infected with the XJ strain at MOI 1. Infection was monitored by immunofluorescence.

Results

Susceptibility to i.p. infection

As shown in Table 1, resistance was gradually acquired with age for both viral strains. Four-day-old animals proved to be resistant to XJCL3 inoculation, while low mortality (~10 %) was recorded with the pahogenic XJ strain; and at 6 days of age, mice became resistant also to the latter. Accordingly, 4 days of age was considered the earliest time resistance became patent, so work was carried on with sensitive 1-day-old mice and resistant 4-day-old mice.

Si treatment

In spite of the macrophage toxicity of this batch of Si, results in Table 2 show that Si pretreatment in both suckling and adult mice failed to modify either the course of the disease or final mortality, regardless of viral strain and of the animals age.

Immunofluorescence (IF)

Since "*in vitro*" studies have shown (González *et al.*, 1982) that JV readily replicates in murine macrophages and is released from them, the "*in vivo*" macrophage-virus interaction was also investigated.

Table 1. BALB/c mouse susceptibility to intraperitoneal inoculation with XJ or XJCL3 strains of Junin virus

Age in days	XJ	XJCL3
1	44%[a] (11/25)[b]	48%[a] (12/25)[b]
2	47% (8/17)	54.54% (12/12)
3	23.53% (4/17)	50% (6/12)
4	11.11% (2/18)	0% (0/22)
5	12.50% (2/16)	—
6	0% (0/18)	—

10^3 LD_{50} were inoculated at the indicated days of life by intraperitoneal route.

[a] percentage of total mortality

[b] number of deaths/total number of animals

Table 2. Effect of silica pretreatment on BALB/c mouse mortality

Age (in days)	Viral strain	Number of animals	Silica dose (mg)	Route	Total mortality (%)	50% mortality[a] (days p.i.)
1	XJCL3	36	5	i.p.	30.55	14
		28	—	—	39.28	15
	XJ	15	5	i.p.	53.33	16
		10	—	—	50	16
4	XJCL3	19	5	i.p.	5.26	15
		19	—	—	5.26	16
	XJ	13	5	i.p.	15.38	16
		23	—	—	26.08	16
45	XJCL3	20	50	i.p.	0	—
		10	—	—	0	—
		14	3	i.v.	0	—
		9	—	—	0	—
	XJ	17	50	i.p.	0	—
		10	—	—	0	—
		16	3	i.v.	0	—
		9	—	—	0	—

10^3 LD_{50} of each strain was given 2 hr later by the same route as silica.
[a] 50% refers to the total number of deaths during the observation period

One-, 4- and 45-day-old mice were infected i.p. with 10^3 LD_{50} XJ strain. From 24 hr to 6 days p.i., adherent PCs were obtained daily without pooling and processed for immunofluorescence. Table 3 shows that viral antigen was detected in most animals at various intervals. In suckling mice and in 60 % of adults, immunolabelling was negative at 24 hr. As from the second day p.i. viral antigen assay became positive as illustrated in Figures 1 and 2.

C3 receptor assay

Since the expression of C3 receptor has been associated with macrophage maturation (Bianco and Pytowski, 1981), activation (Bianco *et al.*, 1975), and inflammation (Walker and Yen, 1982), this marker was chosen to monitor cell alterations. In unstimulated controls, the C3 pattern agreed with results reported by Lu *et al.* (1979), as the percentage of negative cells was below 5 % regardless of the animals age.

Table 3. Immunolabelling of peritoneal macrophages harvested at various times post-infection from BALB/c mice infected at three different ages.

Days p.i.	Age at inoculation					
	1-day-old		4-day-old		45-day-old	
	No. positive[a]/ total number	Infected[b] cells (%)	No. positive[a]/ total number	Infected[b] cells (%)	No. positive[a]/ total number	Infected[b] cells (%)
+1	0/5	0	0/5	0	2/5	15
+2	1/5	20	4/5	90	3/5	80
+3	4/5	90	4/5	70	4/5	90
+4	3/5	90	4/5	90	5/5	90
+5	3/5	50	3/5	90	3/5	80
+6	2/5	40	3/5	80	3/5	80

a) animals with immunofluorescence positive macrophages/total animals number
b) estimated percentage of infected macrophages in positive samples
Mice were inoculated with 10^3 LD_{50} of Junin virus (XJ strain) by intraperitoneal route at the designed age.

Table 4. Percentage of negative C3 cells in peritoneal macrophages harvested at various post-infection intervals from BALB/c mice infected at three different ages

Days p.i.	1-day-old mice		4-day-old mice		45-day-old mice	
	virus[a]	control[b]	virus[a]	control[b]	virus[a]	control[b]
+1	21.25 ± 7.31	9.42 ± 2.86	9.30 ± 3.66	18.50 ± 9.44	10.67 ± 5.54	3.00 ± 0.57
+2	11.08 ± 3.88	10.58 ± 5.15	82.30 ± 3.77	0 ± 0	23.83 ± 8.08	23.83 ± 8.08
+3	19.17 ± 6.74	2.17 ± 1.16	25.20 ± 9.79	4.83 ± 3.44	29.50 ± 3.04	14.00 ± 1.49
+4	6.70 ± 2.54	1.83 ± 1.58	12.40 ± 6.44	8.83 ± 4.63	23.83 ± 6.80	7.75 ± 4.75
+5	0.67 ± 0.66	2.25 ± 0.77	4.33 ± 1.93	3.07 ± 1.08	34.83 ± 9.32	9.50 ± 2.49
+6	7.33 ± 1.16	2.25 ± 0.77	3.40 ± 2.42	5.00 ± 1.19	5.50 ± 2.75	0.75 ± 0.75

[a] animals received 10^3 LD_{50} of Junin virus (XJ strain) intraperitonealy at the designed ages.
[b] animals received normal brain mouse homogenate diluted as the same form as the viral stock, at the designed ages.
Values are expressed as mean ± S.E.M.
At least 3 mice were used for each determination.

I.p. inoculation with virus or even with the normal suckling mouse brain homogenate in equal dilution, led to a drop in C3 percentage, but marker kinetics depended on the host age (Table 4). Since variations in marker expression may be due to macrophage alteration by virus and/or an inflammatory process which may attract C3 negative cells, the assay was carried out on "*in vitro*"-infected cells to rule out the latter possibility.

Accordingly, cells coming from 1-, 4- and 45-day-old animals were cultured for 24 hr and then infected with the XJ strain (MOI : 1). The C3 marker was determined at 1, 24, 48 and 72 hr p.i. In each case, C3 cell labelling ranged from 98 to 100 %, without obvious cytophatic effect (Figure 3).

Discussion

The role of macrophages in viral infections has been widely accepted, as well as their decisive influence on the course of infection. Thus, in many viral diseases correlation between replication ability of a viral strain and pathogenicity has been found (Mogensen 1979, 1984).

In JV experimental infection of *Calomys musculinus*, Si pretreatment has protected the suckling cricetids and rendered the adults partially susceptible to an otherwise harmless pathogen (Coulombié *et al.*, 1986). When the guinea pig, commonly used to distinguish the attenuated strains of JV, was infected, cells of the same lineage exhibited various degrees of virus-strain-dependent susceptibility. Whereas the pathogenic XJ strain was able to infect both macrophages and dendritic cells, the attenuated XJCL3 strain infected only the latter (Laguens *et al.*, 1983). Recently, the rat attenuation marker was described: in lethal XJ infection macrophages played a key role acting as the main replication sites and Si pretreatment conferred protection to the newborn; but in 11-day-old rats, Si treatment led to increased susceptibility attributable to macrophage maturation. However, XJCL3 strain invades fewer macrophages and Si treatment fails to alter the classic course of rat disease (Blejer *et al.*, 1986).

As far as the mouse model is concerned, "*in vitro*"-infected macrophages are known to support viral replication, releasing high virus yields without affecting opsonized SRBC intake, with apparent lack of cellular damage (González *et al.*, 1982). In our "*in vivo*" work, 1- and 4-day-old mice were chosen as susceptible and resistant models, respectively, since the latter showed almost no mortality at that age. Si treatment effect on susceptibility proved negligible regardless of the viral strain, the age of the host and the route chosen. Initially, it seemed there was a quite different behaviour to that observed "*in vitro*", where macrophages are target cells; but IF studies clearly showed "*in vivo*" replication within these cells, regardless of the mouse susceptibility. Thus, macrophages should become incapable of preventing viral spread. In support, some viral diseases, as in ectromelia and mouse cytomegalovirus (Mogensen, 1984), the agent was able to multiply both in macrophages from resistant or susceptible mice.

Nevertheless, as Si blockade was ineffective and IF findings confirmed multiplication, it is suggested that macrophages may hardly be regarded as

essential replication sites for the virus, in contrast with the rat and *Calomys* models for JV or in MHV3 infected mouse (Schindler *et al.*, 1984).

In addition, after i.p. mouse inoculation the C3b marker assays showed a drop in positive cells, indicating that the virus induced inflammation since the "*in vitro*"-infected cells showed 98—100 % C3 positivity. An age-dependent XJ-induced pattern was observed which may partially be accounted for by progressively greater capability of response to inflammatory stimuli, but high IF levels both in suckling and adult mice ruled out the acquisition of any antiviral activity. No C3-mediated phagocytosis at all could be detected in any sample, thus disqualifying any potential activation. The disappearance of macrophages from the infection site is not inconsistent with the persistence of inflammation, as suggested by van Furth (1980).

Results presented here suggest a secondary role for the mouse phagocytic cells in JV infection. Furthermore, our findings stress the need to consider each host-virus model on its own merits, particularly as regards macrophage behaviour. It may therefore be concluded that, following JV infection, each individual host mounts a peculiar strategy which may or may not employ macrophages in its main thrust. Apparently, mechanisms at work in the extraneutrally infected mouse model are considerably more complex than a "macrophage barrier". Further research will no doubt uncover the true means by which the mouse acquires resistance against i.p. Junin virus inoculation.

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Legends to Figures (Plate XXIV):

Fig. 1. Viral antigen in peritoneal macrophages from in vivo-infected 1-day-old mouse at day 4 p.i. detected by immunofluorescence; at bottom a negative cell (630x).

Fig. 2. Viral antigen in peritoneal macrophages from in vivo-infected 4-day-old mouse at day 4 p.i. detected by immunofluorescence (630x).

Fig. 3. C3 rossettes on peritoneal macrophages from *in vitro*-infected adult mouse cells at 48 hr p.i. (250x).