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MACROPHAGE-VIRUS INTERACTION DURING CON A-INDUCED PROTECTION AGAINST JAPANESE ENCEPHALITIS VIRUS IN INFANT MICE

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Summary. — Eight to ten-day-old mice when inoculated i.p. with Concanavalin A yielded a large number of cells in the peritoneal exudate (PE), the majority of which belonged to the macrophage series. Con A-treated and untreated (control) mice were infected i.p. with Japanese encephalitis (JE) virus. The PE cells of Con A-treated mice showed a higher percentage and greater intensity of virus specific immunofluorescent cells as compared with the cells from control mice. No live virus was detected in the Con A-induced cells. However, traces of live virus were found in the cells of control mice, 3—6 hr after infection. In vitro, peritoneal macrophages from Con A-treated mice showed a higher uptake of the virus (83.3 %) within 18 hr as compared to the cells from paraffin-treated mice (34 %). The protection of Con A-induced mice to JE virus challenge by i.p. route could be due to increased uptake and subsequent inactivation of the JE virus in the peritoneal exudate cells.

Key words: peritoneal macrophages; Concanavalin A; Japanese encephalitis virus; infant mice

Introduction

Intraperitoneal inoculation of Con A into 9—10 days old mice protected them effectively from i.p. challenge with JE virus (Kelkar 1962). Several host factors may interact protecting mice from the lethal effect of JE virus when animals are treated with Con A. In primary infections, non-specific factors are the first line defence. During the first few days of infection, non-specific host defence factors interfere with the early stages of virus invasion like adsorption, multiplication and spread to target organs. Among them, phagocytosis by macrophages appears to be upmost important (Mims 1964; Mogensen 1979).

The objective of the present study was to analyse the role of Con A-activated peritoneal macrophages in inducing resistance to mice in primary JE virus infection.

Materials and Methods

Mice. Swiss albino mice, 8—11 days old, randomly bred at the National Institute of Virology (NIV) Pune, were employed.

Virus. The P 20778 strain of JE virus, isolated from human brain (Webb *et al.* 1964) was used. JE virus stocks were prepared as 10 % infected mouse brain suspension in bovine albumin in phosphate saline (BAPS). The challenge dose of virus was 2—3 log ID_{50}/0.03 ml/mouse.

Cell line. Cloned porcine kidney (PS) cell line originally obtained from NIMR Mill Hill, London (in the 79th passage) was used to detect JE virus in the macrophages.

Con A. Con-A 125 μg/0.05 ml/mouse was injected i.p. at —72 hr, —48 hr and —4 hr. This schedule has been shown to be protective against i.p. challenge with JE virus (Kelkar 1985). Control mice were inoculated with 0.05 ml phosphate buffered saline (PBS) according to the same schedule.

Experimental design for in vivo studies. Mice were allocated to five groups and inoculated as follows: Group A — Three doses of Con A followed by JE virus i.p. 4 hr after the last dose. Group B — Three doses of Con A followed by BAPS i.p. Group C — Three doses of PBS followed by inoculation of JE virus, i.p. 4 hr after the last dose of PBS. Group D — Three doses of PBS followed by BAPS i.p. Group E — Uninoculated mice.

Four mice from each group were sacrificed at 4, 24, 48, 72, 96 and 144 hr. PE cells were obtained and cell counts determined. Blood samples were taken from JE virus infected groups (group A and C) at 18, 24 and 72 hr for viraemia studies.

Peritoneal exudate cells. The PE cells from 3—4 mice were pooled and the total cell count determined. To obtain adherent cells, PE cell suspensions containing $1-2\times10^6$ cells were put into plastic Petri dishes and Leighton tubes containing coverslips and incubated at 37 °C in 5 % CO_2 atmosphere. The cells which attached during the 2 hr after seeding were considered for adherent cells.

Indirect immunofluorescence (FA). Adherent cells obtained from groups A and C were stained with anti-JE immune serum prepared in mice and goat antimouse IgG conjugated with fluorescein isothiocynate (Pharmaceuticals, Inc., Life Sciences Group, Cleveland) and counterstained with Evan's blue (0.03 %).

Assay of virus in peritoneal exudate cells. Adherent cells from the plastic Petri dishes and non-adherent cells in the supernatant fluid from group A and C were lysed by two cycles of freezing and thawing. The lysate was tested for the presence of virus in PS cell cultures.

Viraemia studies. For viraemia assays, blood was collected from JE infected mice and injected in 2 days old infant mice by the intracerebral (i.c.) route.

Assay of interferon (IFN). For IFN assay, peritoneal exudate cell supernatant and adherent cells were obtained from group A and C at different time intervals. IFN was assayed by 50 % plaque reduction technique using vesicular stomatitis virus (VSV) in the LM (mouse fibroblast) cell line (Kedarnath *et al.*, 1983). IFN titres have been expressed as per cent inhibition.

Con-A induced protection in neonatally thymectomized mice against JE virus challenge by i.p. route. Neonatally thymectomized mice were kindly supplied by Division of Laboratory Animals, N.I.V., Pune. These mice were inoculated with three doses of Con A as mentioned before and challenged with 2 log LD_{50} of JE virus by i.p. route in 0.03 ml.

Experimental design for in vitro studies. Con A and liquid paraffin-stimulated PE cells were cultured in 24 well Laxbro plates (R. Bhagwandas and Co., Pune, India); 2×10^6 cells were added to each well. After 24 hr incubation at 37 °C in 5 % CO_2, the adherent cells were infected with JE virus (0.5 PFU/cell). The extracellular virus was assayed at different time.

Results

Induction of peritoneal exudate cells

As indicated in Fig. 1, large number of PE cells was induced by three doses of Con A (groups A and B). The majority of the cells were adherent to plastic surface. The cells were of two distinct types 1) cells 5—12 μm, in diameter and without vacuoles. 2) cells of larger diameter, 12—35 μm, and vacuolated in nature.

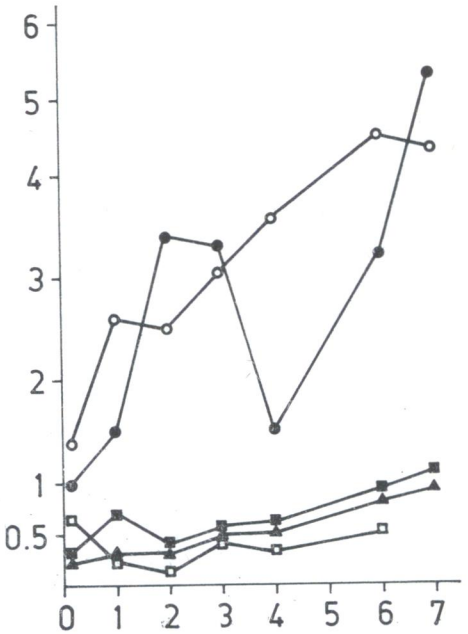

Fig. 1.
Induction of peritoneal exudate cells in mice injected with Con A and JE virus — Group A (○———○); Con A — group B (●———●); PBS and JE virus — group C (□———□); PBS — group D (■———■) and noninfected mice — — group E (▲———▲)
Abscissa: time in days; ordinate: peritoneal exudate cells (10^6/ml)

JE virus interaction with PE cells

In spite of repeated attempts, infectious JE virus could be detected neither in adherent nor in non-adherent cells coming from Con A-treated mice (group A). However, traces of virus could be found at 3 and 6 hr p.i. in macrophages from control mice (group C). No virus could be recovered subsequently suggesting that it did replicate in the macrophages.

Arbitrarily, 20—60 % of macrophages from Con A-treated and JE infected mice (group A) showed JE antigen fluorescence in comparison with 4—10 % of control (untreated infected) mice (group C); the intensity of fluorescence was greater in the Con A-induced macrophages. At 3—6 hr p.i. there was capping of the stain at one or more sites on the cell membrane or, occasionally, in the cytoplasm. At 18 hr p.i. most of the cells showed ring-shaped fluorescence involving the entire cell periphery. Many clumps of macrophages were also present (Fig. 2-I). Nuclear fluorescence appeared at 24 hr p.i. usually located centrally but occasionally at the periphery of the cell. By 72 hr p.i. the cytoplasm became positive for JE antigen so that some cells showed positive fluorescence in nucleus as well as in cytoplasm. At 96 hr p.i. the entire cell showed fluorescence though the intensity was higher in the nucleus (Fig. 2-II). Positive fluorescence of macrophages persisted for 6—7 days p.i., though the intensity was somewhat reduced. A few mature macrophages developed fluorescent vacuoles from 72 hr probably indicating the presence of destroyed virus.

The level of viraemia was always lower in Con A-inoculated mice than in controls. In Con A-treated mice (group A) virus was undetectable at 18 and

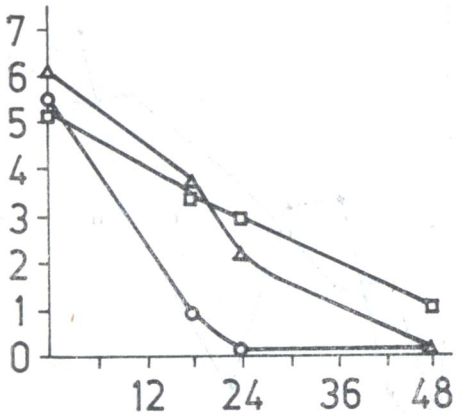

Fig. 3.
Assay of unadsorbed JE virus from the supernatant fluid from Con A induced macrophage culture (○——○), paraffin-induced macrophage culture (□——□), and inactivation curve of JE virus in the absence of macrophages (△——△) Abscissa: time in hr; ordinate: $\log_{10}$ PFU/ml

24 hr, but it was present by 72 hr. (2.13 ID_{50}). In control mice (group C) about 2.5 ID_{50} of the virus was detectable at 18 and 24 hr and by 72 hr the virus titre was 3.63 ID_{50}. A single dose of Con A (125 μg) does not protect mice against JE virus. Following a single dose of Con A at 18 hr there was a slight reduction in viraemia to a titre of 2.0 ID_{50} which later rose to similar levels as found in controls.

IFN assay

Con A-induced macrophages (group A) showed low but detectable IFN level at the time of virus inoculation (76 % inhibition of VSV) but IFN could not be detected in macrophages from unstimulated mice.

Studies on virus macrophage interaction in vitro

Macrophages from Con A-treated mice phagocytosed the virus most effectively (Fig. 3). Within 18 hr about 83.3 % of the virus was engulfed and by 24 hr no detectable virus remained in the supernatant of macrophage cultures coming from Con A-treated mice. However, virus was detectable for 48 hr in the supernatants from macrophage cultures from paraffin inoculated mice. The pattern of virus uptake did not differ significantly from that of the virus inactivation curve.

Protection against JE virus challenge in mice

Neonatally thymectomized mice were protected against JE virus when inoculated with three doses of Con A. It was observed that 79 % mice survived, while controls showed 100 % mortality. Mice, 8—10 days old, inoculated i.p. with three doses of Con A, when challenged with 2 logs of JE virus given subcutaneously instead of by i.p. route were not protected.

Discussion

The present study revealed that Con A-treated mice were protected against JE virus if challenged by i.p. route but not by s.c. route. The reason for such protection appears to be Con A-induced local immunity. As shown here, three (i.p.) doses of Con A, which are protective against JE virus, also induce large number of peritoneal macrophages in infant mice. Con A is known to increase the number of PE cells in adult mice when inoculated i.p. (Raz *et al.*, 1977). Further investigations were made on Con A induced macrophages and JE virus interactions.

Earlier, JE virus antigen was demonstrated by the FA technique in mononuclear cells from mice infected with JE but live virus was rarely detectable (Kelkar and Banarjee 1977). In the present study, we observed that the intensity of fluorescence and the number of fluorescent cells was greater in Con A-stimulated cells as compared to controls. It appears that the uptake of JE virus was more intensive in Con A stimulated cells as compared with the controls. Con A is known to stimulate endocytic activity in macrophages (Edelson and Cohn 1974). Probably, increased JE virus uptake is due to endocytic activity. JE virus growth was enhanced in vitro in Con A--treated PS cell line due to increased virus adsorption (Kelkar 1985).

Although Con A induced cells showed bright fluorescence, no live virus could be recovered from them. This is suggestive of abortive growth cycle of JE virus in Con A-induced cells. Abortive growth cycles have been also reported for several viruses by other authors (Buchmeier *et al.*, 1979; Daniels *et al.*, 1978). Further work to locate JE virus specific polypeptides in these cells by immunoprecipitation is underway. The JE virus specific fluorescence was visible for prolonged period only in the smaller cell population which did not have vacuoles. It can be inferred that there was some degree of antigen synthesis in these cells. In the larger cells with vacuoles the viral fluorescence was only transiently visible within the vacuoles. It appears therefore that the mature cells with vacuoles destroyed the virus readily with the help of lytic enzyme present within the vacuoles.

Mouse peritoneal macrophages are an inefficient host for the growth of both alpha and flaviviruses (Groen *et al.*, 1976). Among flaviviruses, although yellow fever and West Nile viruses multiply in mouse macrophages (Seamer 1965), JE virus does not multiply in cultured adult mouse peritoneal macrophages (Gogate and Nayer 1976).

Immunomodulators that cause an influx of macrophages into the peritoneal cavity enhance resistance to several experimental viral infectious (Larson *et al.* 1972; Shayegani *et al.* 1974; Starr *et al.* 1976; Glasgow *et al.* 1977; Kirchner *et al.* 1977). Our data support the possibility of a nonspecific activation of macrophages by Con A which results in resistance to JE virus. This is substantiated by the finding that neonatally thymectomized mice were also protected with Con A. Whether macrophages are the only barrier to JE virus infection, and whether other factors like interferon and natural killer cells when stimulated by Con A play a role in the virus clearance need to be further investigated.

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Explanation to Figures (Plate XXVIII):

Fig. 2. Photomicrographs of Con A-induced adherent peritoneal exudate cells showing JE virus antigen stained by immunofluorescence at 18 hr p.i., magn. × 400 (I) and at 96 hr, magn. × × 200 (II).