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# DEVELOPMENT OF SPECIFIC IMMUNE RESPONSE IN MICE INFECTED WITH JUNIN VIRUS

*H. A. BARRIOS, **S. N. RONDINONE, *J. L. BLEJER, **O. A. GIOVANNIELLO, **N. R. NOTA

Chair of Microbiology, Parasitology and Immunology, Faculty of Medicine, University of Buenos Aires, 1121 Buenos Aires, Argentina

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*Summary.* — Different parameters of specific immune response involved in the resistance to intracerebral Junin virus (JV) infection were studied in adult BALB/c mice. The relationship of virus replication to production of antiviral antibodies, to occurrence of cytotoxic T cells and to development of delayed-type hypersensitivity response was evaluated. Spleen cytotoxic T cells were assayed by $^{51}Cr$-release method on virus-infected H2 compatible targets. Effector T cells were detected on day 2, reached peak concentrations by day 6 and declined on day 10. These cells seemed responsible, at least in part, for virus clearance from the infected target organ, since virus could not be recovered from the brain in any sample taken on days 2, 5, 6, 8, 10, 15 and 20 post infection (p. i.). All three main antibody classes common in viral infection were present. Serum antibodies appeared later than the T cell cytotoxic response. Neutralizing antibodies and those detected by immunofluorescence prevailed in the IgG fraction, whereas the IgM antibody class was reactive in complement fixation assay. Challenge of infected mice with JV did not result in production of delayed-type hypersensitivity as measured by footpad swelling irrespective of the route of sensitization. The possible interpretations of these findings are discussed in connection with the resistance of adult mice to JV infection.

*Key words*: *Junin virus*; *cell mediated cytotoxicity*; *delayed type hypersensitivity*; *antibody production*; *mice*

## *Introduction*

The immune response to a number of viral infections has been shown to consist of a cellular and humoral component, which relative contribution seems to vary with the infecting virus as well as with the host.

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Although it was demonstrated that the humoral immune response is critical in recovery from a number of experimental primary viral infections (Griffin and Johnson, 1977), in recent years increased emphasis has been placed on the importance of cellular immunity in recovery process (Kraaijeveld *et al.*, 1979*a*; Yap *et al.*, 1979). Among the manifestations of cellular immunity in viral infection, the capacity of specifically sensitized T lymphocytes to destroy virus-infected target cells in vitro has been shown to correlate with elimination of infectious virus in vivo suggesting a direct role for cytotoxic T lymphocytes in the recovery (Blanden and Gardner, 1976; Kees and Blanden, 1976). Another component of the cell-mediated immune response to viral infection is the delayed-type hypersensitivity (DTH) reaction which can be reliably measured by footpad swelling (Hudson *et al.*, 1979; Kraaijeveld *et al.*, 1979*b*).

There is evidence that these responses are mediated by two distinct subpopulations of T cells (cytotoxic activity by Tc; DTH activity by Td). However, the production of cell population possessing Td activity but devoid Tc activity occurs only under certain experimental conditions (Leung *et al.*, 1980).

JV (a member of Arenavirus group) produced a lethal meningoencephalitis when inoculated intracerebrally (i.c.) into newborn mice (90—100% mortality), while most older animals survived (Boxaca *et al.*, 1973).

Immune reactions were found to play an important role in the recovery from viral infection of adult mice infected with JV. This is based partially on the observation that administration of anti-thymocyte serum or cyclophosphamide significantly increases the mortality of adult mice infected with JV (Giovanniello *et al.*, 1980). Furthermore, other studies have shown that immune serum can protect against otherwise fatal infection of either newborn mice or cyclophosphamide-treated adults (Nejamkis *et al.*, 1975; unpublished).

In view of these findings a more detail study of the immune response of adult mice infected with JV should provide further information concerning the role of immune mechanisms in the recovery. This report examines the humoral response in infected mice, the kinetics of effector Tc cell production in spleen and the appearance of DTH as related to viral growth in brain.

## *Materials and Methods*

*Animals.* BALB/c and C3H inbred mice were used throughout. Mice of same sex and age (45 days old) were used in each experiment.

*Virus.* Junin virus (JV) prototype strain XJ (Catalogue of Arthropod-Borne Viruses, No. 77) with a titer of $10^{7.4}$ $LD_{50}$ per 100 mg of wet brain weight was employed.

*Cell-mediated cytotoxicity assay.* C3H mice were infected by intraperitoneal (i. p.) route with $5 \times 10^3$ $LD_{50}$ JV. Spleens from donor mice were harvested at different times after sensitization; the cell samples prepared by standard procedures were tested for their cytotoxicity.

The $^{51}Cr$ release cytotoxicity assay was carried out as described by Marker and Volker (1973) with minor modifications. Briefly, L-929 target cells were infected with JV at multiplicity of infection (MOI) of 0.1 $TCD_{50}$ and incubated at 37 °C for 48 hr. On the day of the assay, cells were labelled with $^{51}Cr$ at 37 °C for 1 hr and washed thoroughly to remove the excess $^{51}Cr$.

Thereafter, cell cultures were overlayed with the test cells supension and incubated for 18-20 hr at 37 °C. Spontaneous $^{51}Cr$ release from target cells incubated with the medium only ranged from 4—12%. Percentage of sepcific $^{51}Cr$ release awas obtained by means of the formula:

$$\frac{\text{test counts} - \text{spontaneous release}}{\text{water lysis counts} - \text{spontaneous release}} \times 100$$

All values represent the mean percentage of specific $^{51}Cr$ release from four replicate tubes.

*Footpad swelling measurement.* BALB/c mice were sensitized by various routes (intravenous; footpad; i. c.; i. p.) with $10^3$ $LD_{50}$ JV. Seven or 10 days later, 10% suspensions from both normal and infected mice brains were prepared in Hanks' solution and injected separately into the right hind and left hind footpads respectively. To obtain a baseline control, the thickness of both hind footpads was measured one day before challenge with a pair of calipers (Oditest H. C. Koplin, Schlüchtern-Hessen, Germany, calibrated to $< 0.05$ mm).

The increase in footpad thickness was calculated as the mean value of the differences in thickness after and before challenge (Student Test).

*Studies on humoral immunity.* The appearance of neutralizing (Nt) and complement fixing (CF) antibodies as well as antibodies detected by immunofluorescence (IF) was followed in BALB/c mice infected i. c. with a single dose of $10^3$ $LD_{50}$ of JV. Groups of animals were bled at different times post infection (p. i.) by severing the brachial artery and sera from five to eight animals were pooled. Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) column chromatography was performed applying 1 ml serum (sampled 15 days p. i.) to a 2.5 cm × 95 cm bed in

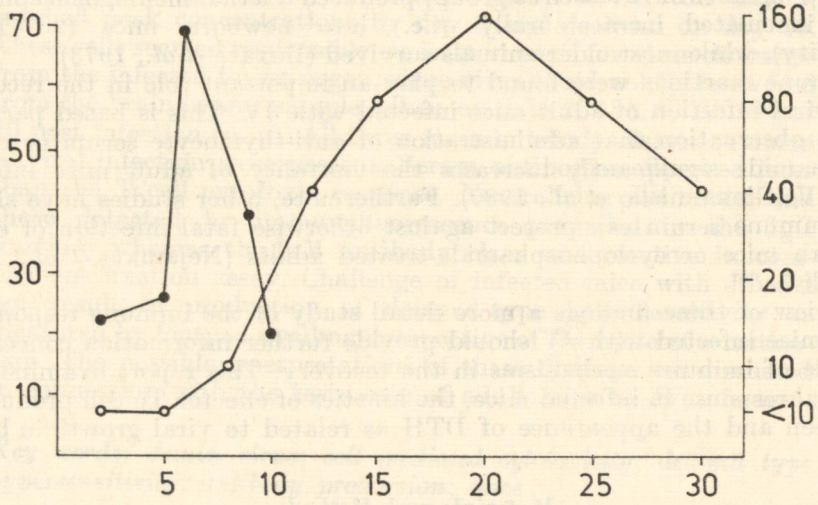



**Fig. 1.**

Course of cytotoxic activity (Tc) of spleen T cells and development of neutralizing antibodies in mice infected i. c. with $10^3$ $LD_{50}$ of JV

Spleen cell to target cell ratios were 40 :1. Each point represents the average values of 6—9 animals in $^{51}Cr$ release test or a minimum of 20 mice in antibody assay.

All values of specific release were found to be significantly different (Student's test) from those obtained with cells from normal animals on infected targets (on days 2, 6, 9 and 10 p.i., $p < 0.001$; day 5 p. i. $0.01 > p > 0.001$).

●——● spleen cells; ○——○ neutralizing antibodies

Abscissa: days p. i.

Left ordinate: $^{51}Cr$ release index (per cent).

Right ordinate: Nt antibody titre reciprocals.

**Table 1. Antibody titres in sera from mice infected with $10^3$ $LD_{50}$ of JV**

| Days p.i. | Antibody | | |
|---|---|---|---|
| | CF | Nt | IF |
| 2 | 1 : 4 | < 1 : 10 | < 1 : 10 |
| 5 | 1 : 4 | < 1 : 10 | < 1 : 10 |
| 8 | 1 : 32 | 1 : 10 | < 1 : 10 |
| 12 | 1 : 64 | 1 : 40 | 1 : 20 |
| 15 | 1 : 64 | 1 : 80 | 1 : 40 |
| 20 | 1 : 64 | 1 : 160 | 1 : 80 |
| 25 | — | 1 : 80 | 1 : 20 |
| 30 | 1 : 32 | 1 : 40 | 1 : 10 |

Types of antibody:
CF: complement fixing
Nt: neutralizing
IF: detected by immunofluorescence
Average values from 5 experiments with at least 20 mice on each day.

phosphate-buffered saline (PBS) at flow rate of 0.3 ml/min. Fractions (6 ml each) were collected and their antibody titers were determined. Nt antibody was measured by the constant virus-serum dilution technique in Vero cells; CF antibody was determined by microtitration technique; IF antibody was measured by the standard indirect technique in infected BHK cells.

*Virus titrations.* Brain homogenates were prepared with Hanks' balanced salt solution. The virus content was determined by standard procedures in Vero cells (Boxaca *et al.*, 1973).

## *Results*

### *Kinetics of cytotoxic T cell appearance in spleen*

Spleen cells were obtained from C3H mice infected with JV 2, 5, 6, 8 or 10 days previously, and assayed in JV infected L 929 target cells. C3H mice and L 929 cells share the H-2 haplotypes (H-2K).

Average levels of Tc activity from 3 of these experiments are shown in Fig. 1. It may be seen that significant activity was already detectable 2 days after infection (cytotoxic index 23.7). It then increased rapidly to a maximum on day 6, when a cytotoxic index of 69.9 was reached. Activity then fell at a similar rate reaching a cytotoxic index of about 20 on day 10.

The time course for the Tc response to JV given above roughly followed that described for other viral infections (Blanden and Gardner, 1976; Marker and Volker, 1973).

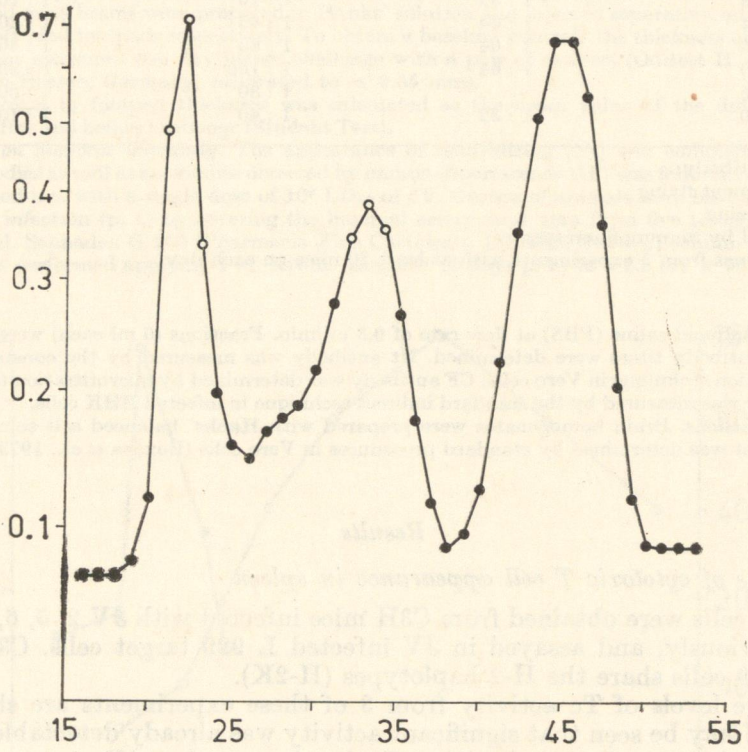
### *Footpad swelling*

The experiments were designed to investigate the level of DTH developing in mice after priming with $10^3$ $LD_{50}$ of JV by various routes of infection.

Challenge of infected mice with JV did not result in increased DTH as measured by footpad swelling irrespective of infection route. Delayed footpad reactions were neither detected at the first (7 days p. i.) nor at second (10

days p. i.) elicitation. Statistical analysis by the Student test showed that the recorded values were not significant ($0.5 > p > 0.1$).

The difficulty of detecting DTH by footpad swelling is noteworthy, since spleen cell populations from animals in the same stage of infection contain high Tc activity.




| | 21 | 22 | 23 | 32 | 33 | 34 |
|---|---|---|---|---|---|---|
| CF | 64* | 1 28 | < 4 | < 4 | < 4 | < 4 |
| Nt | < 10 | < 10 | 40 | 20 | 80 | 40 |
| IF | < 10 | < 10 | < 10 | < 10 | 40 | 10 |

* Titre reciprocals

**Fig. 2.**

Representative separation of immune mouse serum by Sephadex G-200 gel filtration
Animals inoculated i. c. 15 days before bleeding with $10^3$ $LD_{50}$ of JV
Fractions 21—23 and 32—34 indicated (○) were tested for different types of antibodies by complement fixation, by neutralization and by immunofluorescence tests.
Abscissa: fraction number; ordinate: optical density at 280 nm.

*Studies on humoral immunity*

As expected, JV infection induced production of Nt, CF and IF antibodies. The kinetics of antibody response is presented in Table 1. The results obtained can be summarized as follows: Nt and CF antibodies appeared one week after infection reaching a peak between 12 and 20 days p. i. and then declined by 30 days.

As far as IF antibody was concerned, a transient serum level was observed from 12 to 25 days p. i. with a sharp drop 5 days later.

Fig. 2 shows typical Sephadex G-200 chromatography separation of immune mouse serum sampled 15 days p. i. in order to determine Nt, CF and IF antibody activities in each immunoglobulin fraction. Nt antibody predominated in the IgG fraction, whereas the IgM was reactive in the CF assay. Antibodies detected by IF proved independent from both Nt and CF ones and belonged to the IgG class.

*Virus content in brain*

Virus was not recovered from infected adult mice in any sample taken at 2, 5, 6, 8, 10, 15 or 20 days p. i.

## *Discussion*

The relationship of JV and immune response in vivo offers several problems at present not fully understood. When newborn mice were inoculated by i. c. route, virus was detected in brain from the 3rd day until death but without specific antibody production. On one hand, illness and death of newborn JV infected mice is a T cell-mediated effect (Schmuñis *et al.*, 1967; Nota *et al.*, 1976), on the other hand, passive humoral immunity conferred to suckling mice a high degree of resistance (Nejamkis *et al.*, 1975).

These results led to the hypothesis that sensitized T lymphocytes were responsible for death, whereas antibodies were mainly involved in protection and recovery of newborn mice from primary infection.

A totally different set of conditions exists in adult mice: when 45-day-old mice are inoculated by i. c. route, mortality reaches only 8–10% and treatment with antithymocyte serum increases mortality to 28.9% (Giovanniello *et al.*, 1980) suggesting the protective role of T cells. However, our attempts to obtain protection by adoptive transfer of spleen cells from adult donor mice given a single dose of JV, were neither successful in newborns (Giovanniello *et al.*, 1978) nor in cyclophosphamide-treated adult recipients (unpublished).

The possibility that i. c. infected adult mice were unable to become sensitized to JV is questioned in this paper. The results of the present work demonstrated that adult mice exhibited a readily detectable Tc response as early as 2 days p. i.; the response of the spleen cell population culminated by day 6. Recent studies indicate that T cell-mediated cytotoxicity could well contribute to retardation of virus growth and its spread within target

organs (Hirsch *et al.*, 1979; Kraaijeveld *et al.*, 1979*a*). The content of JV in the brain of adult mice infected by i.c. route has proved low or the virus was absent at all. In this regard, the presence of Tc cells and antiviral antibodies suggests that virus administration by i. c. route produces a generalized infection with a systemic immune response, which is perhaps the main factor for the lack of viral replication in the brain, as described in other experimental models (Blanden and Gardner, 1976; Hudson *et al.*, 1979). No special research was conducted on interferon in this paper; as the production of interferon closely parallels JV replication (Boxaca *et al.*, 1973), we consider that its role is secondary. Our findings tend to indicate that the spleen Tc cells become sensitized and reach the CNS at a time when viral replication is undetectable. Tc cells hinder the spread of JV to the brain, but the number of target cells involved would not be enough to cause pathological alterations. This is confirmed by the lack of clinical signs and by mild or undetectable pathology in the brain tissue of infected animals (Nejamkis *et al.*, 1980).

An observation reported here which warrants discussion is the inability of JV to stimulate in vivo DTH in adult infected mice as measured by footpad swelling test. The simplest explanation may be that the antigen-primed effector cells are damaged by JV, or that the mononuclear cells or macrophages are damaged and the accumulation of the latter at the expression site is diminished. This interpretation, however, seems less tenable in our system since it is accepted that the immunopathological nature of the fatal neurological disease induced by JV in newborn mice is a DTH immune response (Taratuto *et al.*, 1973). Therefore, it would be surprising to find that JV should destroy these cells in the adult but not in newborn mice. The results argue strongly against the destruction of T cells in JV infection of mice. A second explanation would arise from mutual immunoregulation between DTH and humoral antibody response (Lagrange *et al.*, 1974; Ramshaw *et al.*, 1976). The fact that in adult JV infected mice antibodies reach a high level would speak strongly for a restraint imposed on the DTH response by feedback inhibition. A third interpretation may be that specific suppressor T cells for DTH could be elicited during JV infection of adult mice (Kaufmann *et al.*, 1980). Their existence was not determined in these experiments and remains to be elucidated.

Although the given results could be interpreted as evidence for the lack of DTH response in adult mice infected with JV, we believe that the level may be too low to be detected by the adopted technique. Recalling the widely studied influenza virus-mice model in which no protocol is known that results in production of Tc but not Td (Peung *et al.*, 1980), perhaps a more accurate method is essential. This proposal is currently being investigated in our laboratory.

Another feature of interest concerns the antibody response involved in JV infection and their possible role. All three main types of antibodies common in viral infection were present, most likely directed against several antigenic components of the virus. Their kinetics and distribution among serum proteins resembled very closely to other antiviral responses (Hotchin, 1971; Griffin

and Johnson, 1977). It is now widely accepted that Nt antibodies are protective by binding to virus-coded proteins on the surface of virus infected cells resulting in cell lysis. This effect would decrease the amount of released virus and benefit the host if these doomed cells were destroyed before maximal production of infectious virus had occurred.

In our case, detectable serum antibodies appeared later than the Tc response and, therefore, it seems that they rather participated in elimination of the residual virus.

With respect to the antibody capable of complement fixing, this could bind to infected cells and lyse them together with the complement; or it could act in accordance with null cells in an antibody-dependent cellular cytotoxicity reaction (Shore *et al.*, 1976).

At the present, we are unable to offer a single explanation of the findings reported in this and previous papers. It should be emphasized that after JV infection of adult mice, Tc cells are generated, which are most probably involved in virus clearance in vivo, although T cell mediated DTH reactions are not detected.

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