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ABROGATION OF JUNIN VIRUS ENCEPHALITIS BY CRITICAL CYCLOPHOSPHAMIDE TIMING AND DOSAGE[1])

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Summary. — Junin virus-induced encephalitis in suckling mouse is a delayed-type hypersensitivity reaction, whose immunopathologic nature has been proven by suppressing the thymus-dependent response. Cyclophosphamide (CY) given at day +6 post-infection (p.i.) has been shown to modulate infection, presumably by T_{DTH} lymphocyte inactivation. To determine critical timing and i.p. drug dose, brain histology and survival were studied in 3-day-old Balb/c mice, inoculated i.c. with Junin virus. Optimal protection was achieved with a non-toxic, 50 mg/kg CY dose at day 6 p.i. (+6): no brain tissue damage was detected in animals killed at day +12, when the necropsied controls exhibited widespread lesions. Other timings (day +3, +4, +5) proved less effective. As regards alternative dosage at day +6, 30 mg was useless, and severe leptomeningitis was evident, whereas 40 mg significantly lowered mortality, and lesions were much milder and less constant. It seems that the 50 mg/kg CY dose must be administered at a critical time p.i. to inactivate sensitized T_{DTH} lymphocytes and to reduce mortality and CNS pathology significantly.

Key words: Junin virus; cyclophosphamide; encephalitis

Introduction

It has been demonstrated that the meningoencephalitis induced in the 1—10-day-old mouse by Junin virus (JV) inoculation, is the outcome of a delayed-type hypersensitivity reaction, known to be the primary cause of brain lesions and death of JV-infected newborn mice. Its immunopathologic

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nature is supported by the protection conferred against JV-induced disease by early thymectomy (Schmunis *et al.*, 1967), by the resistance of nude mice to experimental infection (Weissenbacher *et al.*, 1983) and by antithymocyte serum treatment which was shown to inhibit cell mediated immune disease of CNS (Nota *et al.*, 1976).

In our previous work on this model, we were able to show that 50 mg/kg body weight of cyclophosphamide (CY) administered at day +6 post JV inoculation modified both the time course and outcome of infection most likely due to T_{DTH} lymphocyte inactivation (Giovanniello *et al.*, 1983), whereas no effect could be observed at day +1 (Giovanniello *et al.*, 1980). This paper attempts to pinpoint the critical timing range and minimum dose of a single CY injection required to inhibit T_{DTH} lymphocytes, by correlating drug dose, brain histology and improved survival.

Materials and Methods

Animals. Three-day-old Balb/c mice, raised at the National Atomic Energy Commission (Argentina) Bioterium, hereinafter called newborn, were inoculated intracerebrally (i.c.) with 10^3 LD_{50} of Junin virus, cloned XJ strain, contained in 0.02 ml of Hanks' balanced salt solution.

Cyclophosphamide (CY). The drug (Endoxan-Asta) was dissolved in sterile water immediately before use, and given by intraperitoneal (i.p.) route. Doses were 50, 40 or 30 mg/kg body weight, and timing ranged from 3 to 6 days post infection (p.i.).

Experimental design. Two separate experiments (I and II) were carried out several times.

In I, together 88 animals were used receiving 50 mg CY/kg body weight at days 3, 4, 5 or 6 after JV infection. Once the ideal day had been determined, trial II was performed with 58 mice which received 50, 40 or 30 mg CY/kg body weight on the chosen day. Groups of 5–8 animals each were used throughout.

Two groups having a similar number of control animals were used for each one above: one received CY alone and the other was infected but left untreated. In all cases animals were observed daily to record mortality and neurologic signs for 30 days p.i.

The day p.i. when half of the non-survivors had been found dead was tabulated as "50% mortality day".

Histopathologic studies. Individual samples from appropriate infected groups of trial II were taken at 12 days p.i. in order to assess the brain histopathologic lesions by standard haematoxylin-eosin technique. At random, animals from the same run found dead at any time, were promptly processed for the same purpose.

Statistical analysis. The test equality of two percentages was used; $p < 0.05$ was considered significant.

Results

The effect of various CY doses and schedules on mortality of i.c. JV-infected animals is illustrated in Table 1. As can be seen, the 50 mg/kg CY dose, whatever the timing adopted, delayded "50% mortality day" up to 7 days vs. infected untreated controls (19 or 12–13 days p.i., respectively). A significant drop in final mortality was only achieved when the drug was given at day 6 p.i. (43.5% vs. 70.0% control) (Table 1-I).

In an attempt to achieve protection with a lower dose at day +6, trials were run with 40 and 30 mg CY/kg body mass. As summarized in Table 1-II, with 40 mg there was also a significant decrease in final mortality, but a 30 mg dose had no effect whatever on survival. No drug toxicity effects were seen at any time with the CY schedule employed.

Table 1. Effect of CY treatment on mortality following Junin virus infection in mice[a]

	Cyclophosphamide[b]		$FD/_T$[c]	Mortality	
	Day	Dose (mg/kg)		Total %	50%[d]
I	+3	50	14/21	66.7	18
	+4	50	15/24	62.5	18–19
	+5	50	12/20	60.0	19
	+6	50	10/23	43.5[e]	16–17
	—	—	14/20	70.0	12–13
II	+6	50	8/21	38.1[e]	16
	+6	40	7/17	41.2[e]	17
	+6	30	12/20	60.0	16–17
	—	—	12/19	63.16	14

Each mortality value is the mean of at least 3 determinations. Drug dosage proved non-toxic in control groups (not shown).

[a] 72 hr-old Balb/c mice were inoculated i.c. on day 0 with 10^3 LD_{50} of JV.

[b] CY administered by i.p. route at different times p.i.

[c] Found dead/total number of animals.

[d] Figures represent day p.i. on which 50% mortality of non-survivors was recorded.

[e] Statistical significance $p < 0.05$.

Histologic examination confirmed these findings: brains harvested from untreated controls exhibited severe leptomeningitis with inflammatory exudate and perivascular cuffing, both mainly lymphomonocytic in nature (Fig. 1). In addition, microglial proliferation and neuronal necrosis were found. The findings were similar in animals receiving 30 mg/kg CY (Fig. 2), but the results were rather ambiguous when the dose was raised to 40 mg. In fact, some samples presented congestion without cuffing, while others were hardly affected.

An entirely different picture was seen when 50 mg was administered on day +6, since no tissue damage to CNS could be detected in the samples taken at 12 days p.i. (Fig. 3). In contrast, all brain samples from animals found dead and randomly processed presented lesions similar to controls.

Discussion

For several decades, the alkylating agent cyclophosphamide has been employed to study basic immune response mechanisms against a variety of antigens, including infectious agents. As a rule, CY administration leads to suppression or even tolerance, but injected a few days before antigenic stimulation, the drug enhances delayed-type hypersensitivity (DTH), as well as humoral response. From the beginning, suppression of antibody formation was attributed to elimination of short-lived B lymphocytes (Gabrielsen and Good, 1976; Stockman *et al.*, 1973) while the enhancing effect on DTH and humoral response was ascribed to T suppressor subset dysfunction.

However, the effect of CY on other T cell subsets, particularly DTH effector cells, is far from clear. Following this line of reasoning, our previous experiments have demonstrated DTH effector cells were highly sensitive to CY action "in vivo", since drug administration as late as day +6 during the immune response against sheep red blood cells was able to lower an already established DTH (Rondinone *et al.*, 1983).

In this work we have evaluated the effect of CY in the newborn mouse-JV model, taking the pattern of mortality and brain tissue damage so readily manifest as a measure of DTH reaction. Results for a single 50 mg/kg dose on days 3, 4, 5 or 6 p.i., indicate that no matter whether the T lymphocytes become sensitized to the virus, the drug inhibits their action only at a critical time p.i., namely day +6, when mortality drops over a third of its control value. This conclusion was further supported by abundant clinical signs and the high mortality observed in infected animals receiving treatment at any other interval assayed (66.7%; 62.5%; 60.0%, at day +3, +4, +5 respectively), as well as in controls (70.0%).

Of course, DTH restarts due to cell subset regeneration soon after the drug is metabolized, as patently shown by the delay in "50% mortality day", 4—7 days after controls. Such regeneration following CY treatment was also advanced by Mitsuoka *et al.* (1979) for DTH regulation in mice, to the effect that enhancement of DTH reaction requires adequate recovery of effector T cells from damage induced by CY treatment. Direct proof is available from results obtained by critically timed multiple CY doses (at 4-days intervals) in our model (submitted for publication). As far as CY dosage was concerned, histologic studies appeared to confirm clinical findings. Indeed, brain lesions compatible with DTH reaction were invariably observed in untreated controls and in animals receiving 30 mg CY/kg. Although survival differences were negligible for both the 40 and 50 mg by dose, the latter was chosen as the ideal (nontoxic) dose due to the utter lack of brain tissue damage in animals necropsied at 12 days p.i.

Summing up, the experiments reported here indicate that timing is just as critical as CY dosage in achieving the desired protective effect.

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Legends to Figures (Plates XLIV–XLV):

Fig. 1. Junin virus encephalitis at day 12 p.i. induced in a newborn mouse by i.c. inoculation of 10^3 LD_{50} of JV, featuring mononuclear perivascular infiltration around a small brain vessel, with moderate microglial hyperplasia. H.E., magn. 400×.

Fig. 2. Similar histologic findings at day 12 p.i. in a mouse infected as above, but treated with CY 30 mg/kg at day + 6. H.E., magn. 400×.

Fig. 3. Mouse infected as above, treated with CY 50 mg/kg. Both perivascular cuffing and microglial hyperplasia are entirely lacking at day 12 p.i. H.E., magn. 400×.