INTERFERON PRODUCTION AND IMMUNE RESPONSE INDUCTION IN APATHOGENIC RABIES VIRUS-INFECTED MICE

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Received December 7, 1993; revised February 25, 1994

Summary. – Pathogenic parental rabies virus strain CVS (challenge virus standard) and its apathogenic variant RV194-2 were shown to differ in their ability to induce interferon (IFN) and immune response of the host. After intracerebral inoculation, IFN and antibody production was higher in the RV194-2 virus-infected mice than in the CVS infection. The enhancement of 2-5A synthetase activity, an IFN-mediated enzyme marker, showed biochemical evidence that IFN is active in both apathogenic and pathogenic infections. On the other hand, spontaneous proliferation *in vitro* of thymocytes and splenocytes from CVS virus-infected mice was strongly inhibited in contrast to the RV194-2 infection. In the CVS infection, the thymocyte proliferation was more affected than the splenocyte proliferation. However, in the RV194-2 infection, the thymocyte proliferation was higher than of the splenocytes. These results suggest a better performance of T-cell response to the RV194-2 infection than to the CVS infection. This fact can be critical for an enhancement of antibody production in the apathogenic infection and subsequent virus clearance from the brain of RV194-2 virus-infected mice.

Key words: rabies virus; pathogenic and apathogenic variants; immune response; cell proliferation

Introduction

Rabies virus neurovirulence has been experimentally studied in rats and mice to understand the mechanisms involved in the pathogenesis of the rabies infection (Tsiang, 1979; Gillet *et al.,* 1986; Jackson and Reimer, 1989; Tsiang *et al.,* 1989).

Monoclonal antibodies developed against the glycoprotein of rabies virus isolated and characterized (Wiktor and Koprowski, 1978; Flamand *et al.,* 1980; Coulon *et al.,* 1982*a*; Lafon *et al.,* 1983). Several of them neutralized the CVS strain used to select variants employed in studying the rabies virus neurovirulence (Coulon *et al.,* 1982*b*; Dietzschold *et al.,* 1983, 1985; Kucera *et al.,* 1985; Seif *et al.,* 1985; Jackson, 1991).

The RV194-2 virus, an apathogenic variant selected from pathogenic CVS virus, contains an amino acid substitution at position 333 (Arg –> Ile or Gln) of the glycoprotein molecule. According to the authors, this event is essential for the integrity of an antigenic determinant and for the ability of the rabies viruses to produce a lethal infection in adult mice (Dietzschold *et al.,* 1983, 1985). Recent investigations have showed that the CVS and the RV194-2 viruses spread throughout CNS at similar rates, but the CVS infected many more neurons than did the RV194-2 (Jackson, 1991).

The factors that determine virus clearance in nonlethal rabies infection are not well known, but certainly an efficient immune response of the host may be involved. In fact, it was demonstrated that the high egg passage (HEP) strain of the rabies virus, which produces an inapparent infection in adult mice, becomes lethal when inoculated into immunosuppressed mice (Miller *et al.,* 1978). On the other hand, IFN has been showed to act as a modulating factor not only in the evolution of the rabies disease but also in the enhancement of rabies antibodies production in infected mice (Marcovistz *et al.,* 1986, 1987).

In the present study, we compared the apathogenic RV194-2 virus variant and its parental pathogenic CVS

*Recipient of Brazilian government fellowship CNPq

virus strain for their ability to induce IFN, antibodies and the lymphocyte proliferation in BALB/c mice.

Materials and Methods

Mice. Male BALB/c mice, six-week old, were obtained from Oswaldo Cruz Institute Facilities. Mice were injected ic with 30 µl of virus diluted to 100 PFU/ml of RV194-2 or 100 LD_{50}/ml of CVS, respectively.

Virus. The CVS was prepared as a 20% brain homogenate in PBS from infected mice. The RV194-2 was kindly provided by Dr. Dietzschold from the Wistar Institute, Philadelphia, and produced in BHK-21 cell monolayers in Eagle's Minimum Medium supplemented with 5% foetal calf serum.

Plasma preparation. Mice were bled from the retroorbital plexus and blood was collected in polystyrene tubes containing heparin (100 U/ml). Plasma was collected after centrifugation (200 × g, 15 mins) and stored at -70 °C.

Tissue extracts. Frozen brains were homogenized in a glass Dounce homogenizer in 2 ml of buffer containing 100 mmol/l Hepes pH 7.6, 10 mmol/l KCl, 2 mmol/l Magnesium acetate, 7 mmol/l mercaptoethanol and aprotinin (100 U/ml). The homogenates were left for 15 mins at 4 °C before addition of Nonidet P40 (final concentration 0.5%). Each suspension was then sonicated for 10 secs and centrifugated at 1,500 × g for 25 mins. Brain extracts were stored at -70 °C.

Antibody titration in plasma was carried out by the fluorescent focus inhibition test using Terasaki plates as described by Reagan *et al.* (1983).

IFN titration. IFN titer of plasma or brain extract (without mercaptoethanol and NP 40) was calculated as the reciprocal of highest sample dilution that was capable of inhibiting by 50% CPE of encephalomyocarditis virus on mouse L-929 cells (Marcovistz *et al.*, 1984).

Assay of 2-5A synthetase. The 2-5A synthetase activity brain extracts was assayed in a reaction mixture (600 µl) containing 200 µl of brain extract, 20 mmol/l Hepes pH 7.6, 50 mmol/l KCl, 25 mmol/l Magnesium acetate, 7 mmol/l mercaptoethanol, 5 mmol/l ATP, 10 mmol/l creatine phosphate, creatine kinase (0.16 mg/ml), poly(I).poly(C) (0.1 mg/ml) and 20 µl of ^{3}H-ATP (0.1 mCi/ml, Amersham). Incubation took 90 mins at 30 °C and was stopped by heating at 80 – 90 °C for 5 mins. The ^{3}H-labelled 2-5A was purified by DEAE-cellulose chromatography and the radioactivity of the entire sample was measured. The concentration of 2-5A in AMP equivalents was estimated from the percentage of incorporation of radioactivity from input ^{3}H-ATP into 2-5A (^{3}H-cpm). The 2-5A synthetase levels were calculated on this basis, one unit corresponding to 1 nmol of 2-5A synthesized per mg of protein (Hovanessian and Riciere, 1980).

Statistical analysis. Differences between experimental and control groups were analyzed by Student's t-test for unpaired data.

Splenocyte and thymocyte proliferation. Mice were killed by cervical dislocation and the spleen and thymus was removed surgically and freed of contaminating blood by washing with Hanks' Balanced Salts (HBSS) supplemented with penicillin (500 U/ml) and streptomycin (100 µg/ml). The splenocytes and thymocytes were prepared mechanically by sieving minced organs through stainless steel mesh into RPMI-1640 medium supplemented with antibiotics. The cells were pelleted and the red blood cells eliminated by Tris-buffered ammonium chloride (0.16 mol/l) and then washed twice in ice-cold medium. The cell viability was determined by the Trypan blue exclusion test, and 2×10^6 cells, suspended in 100 µl of RPMI-1640 medium supplemented with 1 mmol/l L-glutamine, 5 µmol/l mercaptoethanol, antibiotics and 10% Controled Process Serum Replacement type 2 (CPSR-2, Sigma), were placed in each well of 96 well-microplates and incubated with 1 µCi/well of ^{3}H-thymidine (^{3}H-TdR) for 6 hrs at 37 °C in 5% CO_2. Thereafter the cells were harvested on glass filters and the ^{3}H-TdR incorporation measured by liquid scintillation counting (Bradley, 1980).

Results

Production and action of IFN

The production of IFN during RV194-2 and CVS virus infections was investigated by titration of IFN activity in plasma and in brain extracts. The 2-5A synthetase activity, a marker for production and action of IFN in cells or tissues, was also analyzed in brain extracts, the target organ of the

Table 1. Kinetics of IFN and 2-5A synthetase activities in plasma and brain of RV194-2-infected mice

Days p.i.	Plasma	Brain	
	IFN (U/ml)	IFN (U/mg protein)	2-5A synthetase (nmol/mg/hr)
Non-infected control	<40	<40	0.3 ± 0.20
1	2133 ± 739	1920 ± 701	1.25 ± 0.21
2	2560 ± 0	2133 ± 739	1.1 ± 0.28
3	2560 ± 0	5120 ± 0	1.25 ± 0.21
4	4266 ± 1478	5120 ± 0	2.0 ± 0
5	5120 ± 0	> 5120	1.7 ± 0.52
6	5120 ± 0	> 5120	1.95 ± 0.21
7	3413 ± 1478	2133 ± 739	1.15 ± 0.07
8	1920 ± 701	2560 ± 0	1.15 ± 0.07
9	640 ± 0	1280 ± 0	1.0 ± 0
10	426 ± 184	640 ± 0	1.11 ± 0.07
11	426 ± 184	640 ± 0	1.35 ± 0.07
12	213 ± 92	320 ± 0	1.0 ± 0.14
13	<40	<40	0.8 ± 0
14	<40	<40	0

Two independent experiments were made and 3 mice were taken for each time interval. Results are expressed as means ± SD.

Table 2. Kinetics of IFN and 2-5A synthetase activities in plasma and brain of CVS infected

Days p.i.	Plasma	Brain	
	IFN (U/ml)	IFN (U/mg protein)	2-5A synthetase (nmol/mg/hr)
Non-infected control	<40	<40	0.3 ± 0.20
1	213 ± 92	426 ± 184	1.1 ± 0.09
2	533 ± 184	853 ± 369	1.6 ± 0.28
3	853 ± 369	1066 ± 369	1.7 ± 0.56
4	1066 ± 369	1280 ± 0	1.4 ± 0.28
5	2133 ± 739	2560 ± 0	1.75 ± 0.35
6	2560 ± 0	2133 ± 739	2.0 ± 0
7	2133 ± 739	2560 ± 0	2.4 ± 0.28

For legend see Table 1.

rabies virus replication (Tables 1 and 2). In RV194-2-inoculated mice, IFN was detectable from day 1 until the day 12 p.i. A peak of IFN activity was found on the days 5 – 6, at a time corresponding to virus replication in the brain. The high levels of IFN in plasma and brain found earlier in the infection may be due to the ic virus inoculation. The shut off of IFN production was found from the day 13 p.i. At that time, there was a total virus clearance from the brain by immunofluorescence assay (data not shown).

The IFN activity in plasma and brain of CVS-infected mice was also detectable 24 hrs after the ic virus inoculation. At the beginning of infection, its level was lower than in RV194-2 infection. In both infections, an increase of the 2-5A synthetase activity was observed concomittantly with the IFN production. In contrast to the RV194-2 infection, the CVS infection was lethal and the animals died with high levels of virus, IFN and 2-5A synthetase activity in their brain.

Table 3. Plasma neutralizing antibody titration in mice inoculated with RV194-2 and CVS viruses

Days p.i.	Antibody titers	
	RV194-2	CVS[a]
2	3.2 ± 0	0.0
3	12.8 ± 0	0.8 ± 0
4	–	3.2 ± 0
5	12.8 ± 0	4.8 ± 2.2
6	38.4 ± 18.0	3.2 ± 0
7	–	4.8 ± 2.2
8	68.13 ± 29.3	–
9	102.0 ± 0	–
10	136.0 ± 59	–
11	204.0 ± 0	–
12	204.0 ± 0	–
13	204.0 ± 72.0	–
14	204.0 ± 72.0	–

Titers are expressed as means ± SD.
[a]On the day 7, all mice inoculated with CVS died.

Humoral immune response

The RV194-2 virus was cleared from CNS of mice around the day 14 p.i., while the CVS virus remained until the death of the animals. It was then interesting to investigate among other immune parameters the neutralizing antibody response in both infections (Table 3). In the RV194-

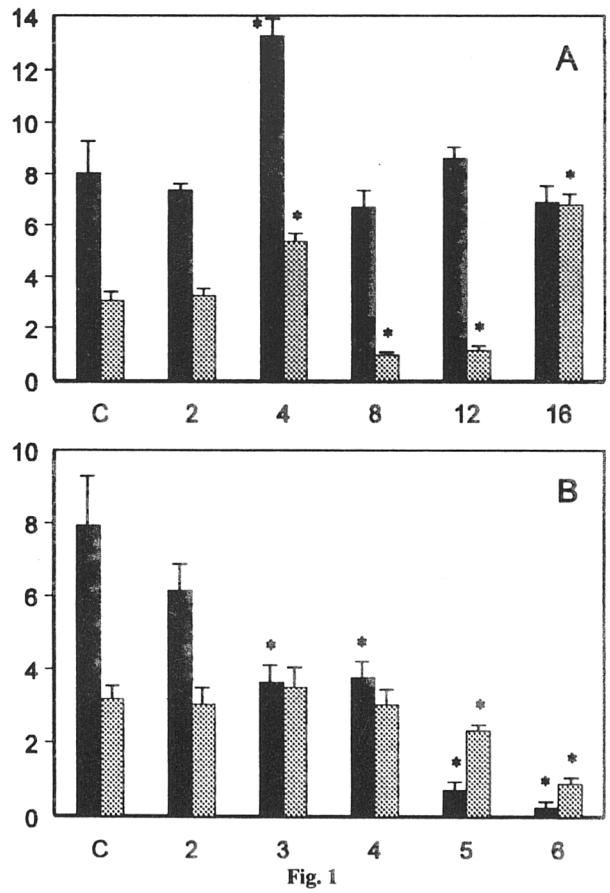

Fig. 1

Spontaneous proliferation of thymocytes and splenocytes from CVS- or RV194-2-infected mice

Thymocyte and splenocyte proliferation from RV194-2-infected (A) or CVS-infected (B) mice. Abscissa: days p.i. (C - control); ordinate: cpm × 10^{-3}. Black (thymocytes) and stippled (splenocytes) columns represent means of 3 separate experiments with SD. * Statisticaly significant ($p \leq 0.05$).

2-inoculated mice, circulating neutralizing antibodies were already noticed on the day 2 p.i. and their level rose rapidly, while in the CVS-inoculated mice the antibody production began to be detectable only on the day 3. The RV194-2 infection always induced higher antibody production than the CVS infection. On the day 7 all CVS-inoculated mice died.

Splenocyte and thymocyte proliferation

The capacity of splenocytes and thymocytes to proliferate spontaneously *in vitro* after inoculation of the CVS or RV194-2 viruses in mice is shown in Fig. 1. While both splenocytes and thymocytes from normal mice were found to proliferate spontaneously, cells from CVS-infected animals proved unable to do so. However, the splenocytes and thymocytes from RV194-2-inoculated mice succeeded in proliferating in the same conditions, with only a temporary inhibition of the splenocytes between the days 8 and 12 p.i.

Discussion

The injection of the apathogenic rabies virus strain RV194-2 into the mouse brain produces nonlethal infection of CNS, and the clearance of the virus occured around the day 14 p.i. However, its parental strain CVS caused lethal infection in mice after inoculation by the same route. Previous studies, employing apathogenic rabies virus strain have shown that the survival of mice is not due to an inherent property of the apathogenic viruses but is due to the ability of the host defense, since suppression of the host immune response produces a lethal infection (Miller *et al.*, 1978; Smith, 1981). In the present study, the ic inoculation of mice with RV194-2 virus resulted in higher levels of IFN in the plasma and brain of these mice than those found in the pathogenic CVS infection. In accordance with these results the level of 2-5A synthetase, an enzyme marker of the presence and action of IFN in organism (Hovanessian *et al.*, 1977; Hovanessian and Riviere, 1980; Baglioni *et al.*, 1980; Laurence *et al.*, 1985), was enhanced during the apathogenic and pathogenic infections. This result brought biochemical evidence that IFN production is active in both apathogenic and pathogenic infections. We have previously demonstrated that IFN plays a role in the defense response of mice against CVS infection, because when these animals were treated with anti-IFN globulin the morbidity period was significantly shorter than that in control mice (Marcovistz *et al.*, 1986). Furthermore, IFN induction has been shown to be an important characteristics of the efficacy of rabies vaccines (Baer *et al.*, 1977; Atanasiu, 1982). These findings lead us to suggest, at least in part, a protective effect of endogenous IFN in mice inoculated with the RV194-2 virus due to its high level at the beginning of infection. Another important fact, which must be taken into consideration regarding the RV194-2 virus clearance is the high level of neutralizing antibodies found early after virus inoculation. Thus, high levels of IFN in the brain and of circulating neutralizing antibodies, associated with modifications in the glycoprotein molecule of the RV194-2 virus, might account for the inability of this virus to disseminate rapidly in the brain, allowing for virus clearance via the immune response of the host. In fact, when the RV194-2-infected mice were immunosuppressed by cyclophosphamide or cyclosporin A, they had low or zero levels of neutralizing antibodies and developed signs of the rabies disease similar to the CVS-infected mice (data not shown).

Despite the undeniable role of the neutralizing antibodies in the virus clearance, the T-lymphocytes may also contribute significantly to host defense against the rabies virus. In fact, the cooperation between T- and B-cells for a good antibody responsiveness to rabies virus has been well documented (Kaplan *et al.*, 1975; Turner, 1976; Dietzschold *et al.*, 1987; Ertl *et al.*, 1989; Perry and Lodmell, 1991). In our experiments, spontaneous proliferation of thymocytes and splenocytes was inhibited in CVS-infected mice in contrast to the RV194-2 infection. It is interesting to note that, in the CVS infection, the thymocyte proliferation was more affected than that of splenocytes, whereas in the RV194-2 infection, the proliferation of thymocytes was higher than that of splenocytes. These results suggest a better performance of T-cell response to the RV194-2 infection than to the CVS infection, and this can be critical for a enhancement of the antibody production and subsequently the virus clearance in the apathogenic infection.

The results presented herein suggest that the capacity of mice to clear the RV194-2 virus from CNS must be due to the activation of their immunological system together with the large IFN production in early stages of the infection.

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