Tropical Medicine, 21 (3), 131−138, November, 1979

Persistent Infection of Avirulent Strain of Rabies Virus in Immunosuppressed Mice

Kumato MIFUNE*,**, Tomoichi NAKAMURA* and Kazuaki MANNEN***.

Department of Virology and Animal Research Center**, Institute for Tropical Medicine, Nagasaki University and Animal Quarantine Service, Nagasaki branch, Ministry of Agriculture, Forestly and Fisheries****.*

Abstract: The mice infected intracerebrally with normally avirulent (HEP-Flury) strain of rabies virus were converted into a lethal infection by immunosuppression. In these mice, both humoral and cellular immune responses were impaired and a persistent infection of the virus was taking place in their brains. Low level of interferon was continuously detected in the brains. When these immunosuppressed mice were passively transferred 7 days after infection, either with anti-rabies antibodies or with immune spleen cells, only those mice given antibodies have recovered from the infection. The mechanisms by which the persistent infection was induced in these mice were discussed.

INTRODUCTION

Little is known about the contributions of immunologic mechanisms in the pathogenesis of rabies virus infection.

Neutralizing antibody produced after preexposure vaccination can effectively protect against subsequent challenge of virus in experimental animals (9). However, such a neutralizing antibody is reported to be ineffective when given to animals after infection (1). Moreover, a phenomenon of "early death", immunopathologic aspects of infection, has been observed in rabies and rabies-related virus infection in which animals with relatively low pre-challenge antibody levels succumbed earlier to challenge than did controls (9, 12).

Recently, Kaplan *et al.* (2) reported the conversion of the avirulent HEP-Flury strain of rabies virus to virulence both in athymic and immunosuppressed mice. Miller *et al.* (8) reported the major role of antibody in the recovery of HEP rabies infection in mice but the data also suggested the contribution of cell-mediated immunity in the pathogenesis of rabies.

Contribution No. 892 from the Institnte for Tropical Medicine, Nagasaki University.
Received for publication, October 24, 1979.

The better understanding of immunologic mechanisms in the pathogenesis of rabies virus infection awaits the further clarifications of these points. The present study was preliminarily undertaken to know the kinds of events occurring in the course of conversion from avirulent HEP rabies infection into virulent infection in immunosuppressed mice.

MATERIALS AND METHODS

Viruses : The HEP-Flury strain of rabies virus (HEP), kindly supplied by Dr. Kondo, National Institute for Health, Tokyo, Japan, was used in the 110th passage level in chick embryo fibroblast cells. This strain is attenuated and not lethal when inoculated intracerebrally (ic) into mice older than 2 weeks. The CVS strain, virulent strain of rabies virus was also used at the 24th passage level in suckling mouse brain.

Mice : Six to 8 week-old inbred C_3H/He mice were obtained from animal laboratory. Infected mice were observed daily for sickness and death for 30 days.

Endoxan (Cyclophosphamide) : This was purchased from Shionogi Co., Ltd. and reconstituted just before use and held in an ice bath until use. When endoxan was used as a single dose, 300mg/kg of bodyweight was given intraperitoneally (ip). In the case of multiple doses, the first dose was 200mg/kg and the successive doses were 100mg/kg.

Virus infectivity assay and reisolation of virus : This was done by fluorescent focus formation method using CER cells in Lab-Tek 4 chamber slides (Miles laboratories) as described previously (10). For reisolation of virus, ten percent of mouse brain suspension were inoculated into CER cells and incubated for 3 days at 37°C in 5% CO_2 atmosphere. The cells were then examined for the fluorescent viral antigens by staining with FITC labelled anti-rabies goat serum. Suckling mouse brain inoculation method was also employed concurrently for the isolation of virus.

Assay of anti-rabies neutralizing (NT) antibody : Serum NT antibody was assayed by rapid fluorescent focus inhibition test using CER cells in Lab-Tek 8 chamber slides as described elsewhere (11).

Interferon assay : Ten percent suspensions of infected brains in phosphate buffer solution (PBS), pH 7.4 were incubated for 18 hr at 37°C with L cells in flat-bottom microculture plate (Falcon 3040). After washing of L cells twice with PBS, the cells were challenged with 100 TCD_{50} of vesicular stomatitis virus (VSV) and incubated for additional 2 days at 37°C. Interferon titer was expressed as a reciprocal of endpoint dilution which inhibited the cytopathic effect caused by VSV by more than 50%.

Cell-mediated cytotoxicity test : The procedures of the test were essentially the same as described previously (7, 13). Briefly, confluent monolayers of murine neuroblastoma cells, grown in 25ml plastic flasks with Dulbecco's modified Eagle minimum essential medium (D-MEM) supplemented with heat-inactivated fetal calf serum (FCS), were infected with plaque purified CVS strain of rabies virus at a multiplicity of infection of 0.5 focus forming unit (FFU)/cell. After incubation for 12 hr at 37°C, the cells were labelled with 50μCi of ^{51}Cr for 1 h at 37°C. Then, the monolayers were washed 3 times with D-MEM, dispersed by gentle pipetting, washed twice by low speed centrifugation, and resupended in D-MEM with 10% heat-inactivated FCS. After seeding these target cells into micro-culture plate at a cell density of 4×10^5 cells/well, immune or normal spleen cell suspension were added to each well to yield an effector cell/target cell ratio of 200/1. After 18 hr incubation at 37°C at 5% CO_2 atmosphere, one hundred microlliters of culture medium from each well were carefully taken to measure the radioactivity. The specific cytotoxic index (CI) was then calculated as follows :

$$CI = \frac{\text{cpm.in experimental wells} - \text{cpm.in control wells}}{\text{cpm.in total uptake} - \text{cpm.in control wells}} \times 100$$

Preparation of spleen cells from infected mice or from immunized mice : Spleens were taken from infected mice at intervals after infection. Spleens from mice immunized ip with 10^7 FFU of HEP-Flury strain of rabies virus were harvested on day 7 after immunization. The single cell suspension of spleen was prepared by standard method. Erythrocytes were removed by treatment with 0.83% NH_4Cl.

RESULTS

Effect of immunosuppression on the mortality of mice infected with HEP virus. Preliminary experiment was carried out to examine when after infection the mice should be treated with endoxan to obtain the highest mortality. Table 1 shows that the mortality of

Table 1. Effect of various treatments with endoxan on the mortality of mice infected with HEP virus

Group	Day treated with endoxan after infection	Mortality
1	-1	1/6
2	0	3/6
3	1	5/7
4	1,5	7/7
5	1,5,10	5/5
6	None	0/5

Mice were infected intracerebrally with 1.5×10^7 FFU of HEP virus. When endoxan was used as a single dose, 300mg/kg of bodyweight was given intraperitoneally. In the case of multiple doses, the 1st dose was 200mg/kg and the successive doses were 100mg/kg.

the infected mice is dependent on the time of treatment with endoxan and the highest mortality occurred in the group of mice which received endoxan on days 1 and 5 or on days 1, 5 and 10 after infection.

Table 2 shows the mortality and the day of death of mice treated with endoxan on days 1 and 5 after infection. Such a treatment of mice with endoxan completely converted a non-lethal HEP virus infection into a lethal infection when compared with the mice infected with HEP virus but no endoxan. Essentially no mortality was observed in mice only with endoxan. From these results, the treatment of mice with endoxan on days 1 and 5 after infection was employed in the following experiments.

Effect of immunosuppression on virus replication and the production of interferon in brain and the development of immune responses. In order to examine what kind of events occur in immunosuppressed mice, mice were infected ic with 1.5×10^7 FFU of HEP virus and treated with endoxan as described above. At intervals, the mice were sacrificed and the blood, spleens and the brains were harvested. The blood was examined for the NT antibody and the spleens were processed for the cell-mediated cytotoxicity test. The brain was divided into 2 pieces and a portion was homogeniezed with PBS to examine for the virus infectivity and interferon content. Another portion of the brain was frozen-sectioned and tested for the presence of viral antigens by fluorencent antibody technique.

As shown in Table 3, in immunosuppressed mice, the viral fluorescent antigens were consistently observed in their brains and the virus was recovered from their brains throughout the experimental periods. Low level of interferon production was also consistently observed in those mice, though fluctuated. The discrepancy, however, was noted between the infectivity of reisolated virus and the amount of fluorescent viral antigens observed in the brain, although the amount of virus and of viral antigens could not be assayed quantitatively in the present study because of very little amount of virus.

In contrast, in the mice not treated with endoxan, the virus reisolation and interferon

Table 2. Effect of immunosuppression with endoxan on HEP virus infection in mice

Expt.	Group	Mortality(%)	Day of death
1	HEP[a)] only	0/5(0)	–
	HEP+endoxan[b)]	5/5(100)	12,16,17,17,19
	endoxan only	1/5(20)	16
2	HEP only	0/6(0)	–
	HEP+endoxan	6/6(100)	15,17,17,18,18,19
	endoxan only	0/6(0)	–

a) Mice were infected intracerebrally with 1.5×10^7 FFU of virus.
b) Endoxan was administered to mice intraperitoneally with 200 mg/kg on day 1 and 100 mg/kg on day 5 after infection.

production in the brain were demonstrated only on day 1 after infection.

The antibody production and the cell-mediated immune response as determined by the *in vitro* cell-mediated cytotoxicity test were suppressed in the mice treated with endoxan. Low level of antibody (1 : 40) was firstly detected on day 16 after infection and the level did not increase thereafter until their death. Whereas, in control mice, the antibody was firstly detected on day 5 after infection. Cell-mediated cytotoxic activity of spleen cells was not observed on day 5 after infection, however, significant activity was demonstrated on day 7 and lasted at least until day 11 after infection. These observations appeared to suggest that a persistent infection is ongoing in the brains of HEP infected and immunosuppressed mice during the periods that immune responses are suppressed.

Effect of administration of anti-rabies antibody and rabies immune spleen cells on the outcome of HEP infected and immunosuppressed mice. To know which defect of immune response is more responsible for the induction of the persistent infection in immunosuppressed mice, adoptive transfer experiments were carried out.

HEP infected and immunosuppressed mice on day 7 after infection were given ip either with 0.2 ml of anti-rabies antibody (NT titer, 1 : 1250) or with 5×10^7 spleen cells from mice immunized ip with 10^7 FFU of HEP virus 7 days before. Mice were observed daily for sickness and death for 30 days.

As shown in Table 4, a significant difference in mortality was observed between the mice given antibody and the mice transferred with immune spleen cells, indicating that antibody can cease the persistent infection but the immune spleen cells can not interfere the infection.

Table 3. Effect of immunosuppression with endoxan on virus growth and production of interferon in brain and on immune responses of HEP virus-infected mice

Group	HEP only						HEP+endoxan (on days 1 and 5)					
Day after infection	1	5	7	11	16	19	1	5	7	11	16	19
Virus reisolation	+	−	−	−	−	−	+	+	+	+	+	+
Viral FA antigen	+	−	−	−	−	−	+	+	+	+	+	+
Interferon in brain	25	5>	5>	5>	5>	5>	25	5	10	5	125	25[a]
Serum NT antibody	5>	20	ND[c]	80	5,760	5,760	5>	5>	ND	5>	40	40[b]
Specific cytotoxic index (%) of spleen cells	ND	-4.0	36.9	21.5	ND	ND	ND	-4.0	-5.5	-2.0	ND	-6.8

a) Reciprocal of endpoint dilution of 10 % brain suspension which inhibited the cytopathic effect of vesicular stomatitis virus by more than 50 %.
b) NT antibody titer by fuorescent focus inhibition test.
c) Not done.

Table 4. Effect of adoptive transfer of anti-rabies antibody and rabies immune spleen cells on the outcome of HEP infected and immunosuppressed mice

Mice were transferred with	No.of mice died / No. of mice tested	Mortality (%)
Antibody[a)]	2/12	16.7
Spleen cells[b)]	10/13	76.9
None	5/5	100

HEP infected and immunosuppressed mice on day 7 after infection were given intraperitoneally either with a) 0.2 ml of anti-rabies antibody (NT titer, 1:1250) or with b) 5×10^7 spleen cells from mice immunized with HEP virus 7 days before.

DISCUSSION

It was demonstrated in the present experiments that a persistent infection took place in the brain of the mice infected intracerebrally with HEP-Flury avirulent strain of rabies virus, followed by subsequent immunosuppression. In these mice, humoral antibody response was suppressed until day 11 after infection. These observations are consistent with the Kaplan *et al.* experiments (2) in nude mice. In our experiments, cell-mediated immune response was also found to be suppressed in these mice as determined by *in vitro* cell-mediated cytotoxicity test although this has been expected since the immunosuppression by cyclophasphamide is reported to suppress nonselectively the functions of T cells, B cells and macrophages (4).

The mechanisms or a trigger by which the persistent infection of the virus was induced in these mice may deserve consideration, although the different role of humoral or cellular immune response can not be clearly demonstrated in this kind of study. In non-immunosuppressed mice, the antibody was already produced on day 5 after infection, however, cell-mediated cytotoxic activity of spleen cells was not observed on day 5 and firstly detected on day 7 after infection. And an adoptive transfer experiment showed that antibody passively transferred to HEP infected, immunosuppressed mice on day 7 after infection could cease the potentiation of infection and the mice were recovered from the infection, whereas the spleen cells harvested on day 7 from rabies immune mice could not cease the persistent infection. In addition, it has been demonstrated that the budding site of HEP virus in the neurons of mouse brain was completely restricted to the intracytoplasmic membranes (6). This finding appears to indicate that HEP infected neurons can not be lysed or cleared by immune lymphocytes unless the virus coded viral antigens other than the structural antigens are formed on the surface of the infected neurons.

These observations are tempting to speculate that humoral immune response rather than cellular immune response plays an important role at least as a trigger to the potentiation of this particular central nervous system infection.

The evidences which demonstrated the development of cell-mediated immunity in rabies virus infection have been accumulated (5, 7, 13, 15, 16). However, whether this cellular immune response really plays a protective role in rabies virus infection has not been evaluated. Further studies especially to this point in more defined system are necessary to reveal the possible contribution of cell-mediated immunity in the pathogenesis of rabies.

Other factors such as interferon and defective-interfering (DI) particles have been shown to contribute to an *in vitro* persistence of rabies virus (3, 14). In the present study, low level of interferon was continuously detected in the brains of HEP infected, immunosuppressed mice and the presence of DI particles were also speculated from the discrepancy of the large amount of viral antigens and low infectivity titer of recovered virus. Al hough further studies are needed, such factors might be possible to contribute to the persistence of HEP virus in immunosuppressed mice.

ACKNOWLEDGMENT

A part of this study was supported by grant in aid for scientific research, No. 248169 from the Ministry of Education, Japanese Government.

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免疫抑制マウスにおける狂犬病ウイルス弱毒株の持続感染

三舟求真人*,**，中邑友一*，万年和明***（長崎大学熱帯医学研究所，ウイルス学部門*，熱帯性病原体感染動物実験施設**，及び農林省動物検疫所長崎分室***）

正常マウスに対しては，致死的感染を起さず不顕性感性に終る，狂犬病ウイルス HEP-Flury 株を，マウスに脳内感染し，引続いてエンドキサン（シクロフォスファミド）で免疫抑制を行うと，これらのマウスは発病し，死亡した．これらのマウスから，経日的に脳，脾臓，血清をとり出し，脳内ウイルス及びインターフェロンの測定，血清中和抗体の測定，脾細胞の標的細胞破壊試験による細胞性免疫応答を調べた結果，免疫抑制されたマウスでは，体液性及び細胞性の両免疫応答が障害されており，脳内でウイルスの持続感染が成立していた．また，マウスの死亡迄，低値ではあるが常時インターフェロンの産生が脳内に認められた．この免疫抑制されたマウスに，ウイルス感染後7日目に，抗狂犬病ウイルス血清あるいは免疫脾細胞の移入を行ったところ，抗体を移入された群のマウスでは，著明な回復が認められたが，免疫脾細胞を移入されたマウスでは殆んど回復が認められなかった．以上の成績から，ウイルス感染後，免疫抑制されたマウスにおけるウイルスの持続感染成立の要因について考察した．

熱帯医学　第21巻　第3号，131−138頁，1979年11月