

Host Defence Mechanisms Against Dengue Virus Infection of Mice

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SUMMARY

Serum obtained from mice 3 to 5 weeks after the third i.p. dose of dengue type 2 virus (DV) protected recipient mice against intracerebral challenge with DV, whereas the serum obtained after 1 and 2 weeks provided minimum protection. Adoptive intravenous transfer of immune spleen cells obtained from mice 1 to 5 weeks after immunization did not protect recipient mice against even a small dose (10 LD₅₀) of DV. Depletion of T-cells by treatment of mice with anti-thymocyte serum did not potentiate DV infection. Development of a cell-mediated immune response (CMI) against DV was noted only at two periods by the leucocyte migration inhibition test (LMI), with borderline values of 20 and 21%. Dengue virus did not cause illness or death in mice when given by i.p. or i.v. routes and this was not affected by pre-treatment of mice with silica to damage local macrophages. It is concluded that humoral antibody plays a critical role in recovery from primary dengue virus infection of mice whereas CMI and macrophages appear to have no protective role.

INTRODUCTION

Dengue virus infection in humans may be associated with haemorrhagic manifestations (Rao & Anderson, 1964; Halstead, 1966; Chaturvedi *et al.* 1970*a, b*). There is a possibility that immunopathological processes determine whether infection with dengue virus results in a benign or a haemorrhagic fever (Halstead, 1966; Hammon, 1966), although our findings on three epidemics of dengue do not support this view (Kapoor *et al.* 1978).

Immune mechanisms play an important role in recovery from virus infections. Humoral antibody and cell-mediated immunity (CMI) may both be active, but in a number of virus infections the role of CMI is considered to be more important (Allison, 1972). A contributory factor in the development of resistance against herpes simplex, yellow fever and Cox-sackie B₃ virus infection is the macrophage (Hirsch *et al.* 1970; Zisman *et al.* 1971; Rager-Zisman & Allison, 1973). Halstead *et al.* (1973) have reported peripheral blood lymphocyte stimulation in monkeys infected with dengue virus. They have also shown that replication of the virus occurs to a greater extent in cells previously exposed to DV than in normal cells (Marchette *et al.* 1976). In our previous study we noted that antibody plays a critical role in recovery from acute DV infection of immunosuppressed mice (Chaturvedi *et al.* 1977). In the present study we investigated the role of CMI, macrophages and antibody in the protection of mice against DV infection.

METHODS

Mice. The study was carried out on male albino mice (Swiss strain) weighing 20 to 25 g (4 to 6 months old).

Dengue virus (DV). Dengue type 2 virus (strain 23085) was obtained from the Virus Research Centre, Poona and had undergone 12 adult mouse brain to brain passages in this laboratory. The strains produced 100% mortality by the intracerebral (i.c.) route in infant and adult mice. The virus used in the experiments was in the form of an infected adult mouse brain pool stored at -70°C .

Titration of DV. Localization of virus in different organs of adult mice, namely brain, heart, liver and spleen, were determined at different days after i.c. inoculation of DV. At autopsy, brain, liver, spleen and heart were removed. The organs were cut into thin slices and repeatedly washed with ice-cold Hanks' balanced salt solution (HBSS) until the wash solution was free of blood. A 10% (w/v) organ suspension was prepared in chilled Eagle's minimum essential medium (MEM) containing antibiotics and 10% heat inactivated new-born calf serum. It was clarified by centrifugation in the cold and serial 10-fold dilutions prepared in MEM were inoculated i.c. into groups of adult mice using doses of 0.03 ml. Mice were observed for 21 days and the LD_{50} was calculated by the method of Reed & Muench (1938).

Antibody titres. Individual mouse sera were assayed for haemagglutination inhibiting (HI) antibody by the technique of Clarke & Casals (1958) using 8 HA units of antigen and 1-day-old chick red blood cells. The freeze-dried antigen of dengue type 2 virus (strain 23085) was obtained from the Virus Research Centre, Poona.

Donor cells for adoptive immunization. Adult mice were given 0.5 ml of a 20% suspension of DV infected mouse brain by intraperitoneal (i.p.) inoculation. Three such doses were given at weekly intervals. At 1, 2, 3, 4 and 5 weeks after the third dose, groups of mice were sacrificed. The spleen cells were teased out in MEM and the cell clumps were removed by aspiration. The cells were washed once and resuspended in MEM and counted in a haemocytometer. In different preparations the viable nucleated cells ranged from 90 to 94% as assessed by the trypan blue dye exclusion test.

Sensitized spleen cells were injected intravenously into normal mice at doses of 1 to 4×10^8 cells. After 24 h they were challenged with varying doses of DV intracerebrally. Control groups received spleen cells from non-immune mice.

Serum for adoptive immunization. From the group of mice used as donors of sensitized spleen cells for adoptive immunization, blood was collected directly from the heart at 1, 2, 3, 4 and 5 weeks after the third dose of DV i.p. The blood was pooled from similarly treated mice and the serum was separated and stored at -20°C until used. The serum was diluted 10, 100, 300 and 500-fold and injected in doses of 0.5 ml i.p. into normal mice followed 24 h later by 10^3LD_{50} of DV i.c.

Effect of adoptive cell transfer on antibody forming cells. To investigate the presence of suppressor cells in the immunized mice the following experiment was done. Mice of group A were given 0.5 ml of 10^3LD_{50} DV i.p. Mice of group B were given the same amount of DV i.p. followed 48 h later by the adoptive transfer of 10^8 immune spleen cells i.v. In mice of group C the DV given i.p. was followed 48 h later by adoptive transfer of 10^8 normal mouse spleen cells i.v. Mice from each group were sacrificed in batches of 4 to 5 daily for 9 days beginning 24 h (in group A) or 72 h (in groups B and C) after infection. The spleens were collected and direct antibody forming cells against DV were counted by the technique of Jerne & Nordin (1963) as described elsewhere (Chaturvedi *et al.* 1977; Tandon & Chaturvedi, 1977). The immune spleen cells were obtained at various periods after immunization as described above

for adoptive transfer. The peak antibody forming cell response was observed after 6 or 7 days and therefore, in another set of experiments, sensitized spleen cells obtained at 3 days and 1 to 5 weeks after immunization were transferred into normal mice as described above and the antibody forming cells were counted 6 and 7 days after DV was given i.p.

Preparation of anti-thymocyte serum (ATS). Antiserum against the thymocytes of Swiss mice was prepared in albino rabbits by the method of Levey & Medawar (1966) as described by Blanden (1970). Briefly, the rabbits were given two successive intravenous injections, 14 days apart, of a single cell suspension of 2×10^8 viable thymocytes. The rabbits were bled 7 days after the second injection. Serum was separated, pooled and stored in small portions at -70°C after inactivation at 56°C for 30 min. The cytotoxic titres against mouse thymocytes and spleen cells were determined in the presence of complement by the technique of Golub (1971). The cytotoxicity was comparable to that of the anti-theta serum (prepared in mice immunized with thymocytes from young $\text{C}_3\text{H}/\text{HeJ}$ mice) obtained from Bionetics, Maryland, U.S.A. Treatment with these sera in the presence of complement killed 41.5% of the spleen cells.

Depletion of T-lymphocytes. The ATS was given in doses of 0.1 ml i.p. on alternate days from 2 days before the 10^2 LD₅₀ i.c. DV inoculation until the 11th day after inoculation (Camenga *et al.* 1974). The control group was similarly treated with normal rabbit serum.

Effect of cyclophosphamide. Mice were given cyclophosphamide (Endoxan-ASTA, Khandelwal Lab. Pvt. Ltd., ASTA-Werke, A-G, West Germany) in a single dose of 250 mg/kg body weight i.p. 24 h after 10^2 LD₅₀ DV was given intracerebrally.

Leucocyte migration inhibition test (LMI). This test was performed by the method of David *et al.* (1964) with spleen cells of mice given DV in various doses by different routes as follows. In mice of group A, 0.03 ml of 10^3 LD₅₀ of DV was inoculated i.c. and mice were sacrificed in batches of 5 at 24 h intervals from day 1 to 10. Mice of group B were given 0.1 ml DV i.p. and group C received 1 ml of 10^3 LD₅₀ of DV i.p. Mice of both these groups were sacrificed daily in batches of 4 to 5 from day 1 to 23 and then at 28 and 35 days. Mice of group D were given three i.p. doses of 0.5 ml of 20% DV infected mouse brain suspension at weekly intervals and the mice were sacrificed 1, 2, 3, 4, 5 and 6 weeks after the last dose.

There was almost no difference in the migration pattern whether live DV antigen or ultraviolet inactivated antigen was used. All the tests were set up in triplicate from each mouse. After incubation for about 24 h at 37°C the migration pattern of the leucocytes was recorded on Whatman No. 1 chromatographic paper with the Camera Lucida, using a magnification of $\times 15$. The area of migration was cut out and the paper was weighed. The % migration inhibition was calculated according to David *et al.* (1964). An inhibition of 20% or more was considered significant (Ortiz-Ortiz *et al.* 1974).

Effect of damage of macrophages. Failure to produce illness in mice by i.p. or i.v. inoculation of dengue virus, as observed in previous experiments, could be due to non-specific resistance by macrophages. Macrophages can be destroyed specifically by silica particles (Allison *et al.* 1966). Therefore, mice of groups A and B were given 50 mg of $2\ \mu\text{m}$ sterilized silica particles (quartz dust) i.p.; mice of groups C and D were given 3 mg silica particles i.v. Mice of groups A and C were challenged with 10^2 LD₅₀ of DV i.p. or i.v. 2 h later.

RESULTS

Distribution of DV in the body after i.c. inoculation

Mice receiving DV intracerebrally remained apparently healthy for up to 4 days. On the 5th or 6th day the fur became ruffled and the back arched. Later, mice became markedly sick and from the 8th day paralysis of the hind limbs set in. All the mice died between 8 and 12 days after inoculation. The distribution of the virus in different organs after intracerebral inoculation of 10^2 LD₅₀ DV is shown in Fig. 1. The virus was found in the brain from the 2nd day, reaching a peak on the 9th and 10th day when the titres were $10^{5.8}$ LD₅₀. The virus was detected from the 4th day in the liver, from the 5th day in the spleen and from the 7th day in the heart.

Effect of adoptive immunization by sensitized spleen cells

Adoptive immunization by sensitized spleen cells, obtained at different periods after immunization of mice, did not affect the outcome of DV infection (Table 1). Transfer of normal mouse spleen cells to control mice also had no effect.

Effect of adoptive immunization by serum

Adoptive transfer of serum, obtained at 1 or 2 weeks after immunization, protected a smaller number of mice (Table 2). At later periods the protection was marked even with a higher dilution of the serum.

Effect of adoptive transfer of sensitized cells on antibody forming cells

Adoptive transfer of immune spleen cells collected 10 days after the immunization markedly suppressed the antibody forming cells against DV in the spleen of recipient mice. The results summarized in Fig. 2 show that the maximum plaque forming cell (PFC) count in such mice was $285/2 \times 10^6$ spleen cells on the 7th day. In mice given normal spleen cells (group C) the peak count was 630 and in untreated controls it was 772 cells/ 2×10^6 spleen cells.

Results of the second set of experiments where PFC were counted after i.v. transfer of immune spleen cells collected after 3 days, 1, 2, 3, 4 and 5 weeks of immunization, are summarized in Table 3. The suppression of PFC continued to occur by transfer of immune cells up to 3 weeks after immunizing the donor, while at later periods the suppressive activity diminished.

Leucocyte migration inhibition test

Only the mice of group B (0.1 ml DV i.p.) showed an inhibition of $20 \pm 1.8\%$ on day 14 and the mice of group D (three doses of 0.5 ml of 20% brain suspension i.p.) showed an inhibition of $21 \pm 2.8\%$ at 6 weeks. At every other period the migration inhibition was not significant with various doses of DV.

Effect of treatment with ATS and cyclophosphamide

The results summarized in Table 4 show that cyclophosphamide treatment reduced the mean survival time but treatment with ATS did not potentiate the virus infection. The ATS treatment *in vitro* rendered 41.5% of spleen cells (T-lymphocytes) non-viable, showing the efficiency of the ATS preparation.

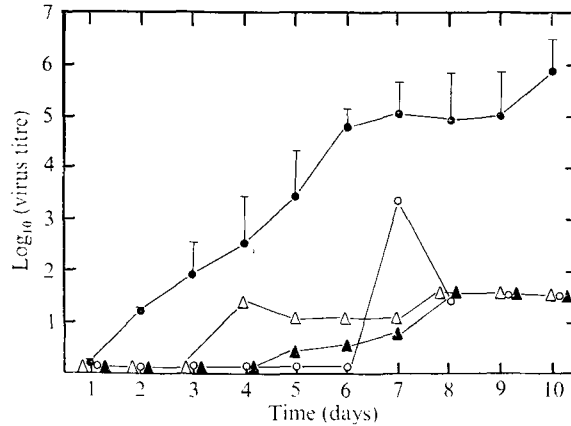


Fig. 1. Virus titres in different organs of infected mice on various days after intracerebral inoculation of DV. Each point represents the mean value for 5 mice for brain tissue and for 2 mice for the other organs. ●—●, Brain; ▲—▲, spleen; △—△, liver; ○—○, heart.

Table 1. Failure of adoptive immunization by spleen cells obtained from specifically immunized mice to protect against intracerebral infection by DV*

Source of cells	Donor cells		DV LD ₅₀ ‡	Mortality	
	Period after immunization	No. of cells† × 10 ⁻⁸		D/T§	Median day of death
Immune cells	1 week	2	10 ⁵	5/5	8.8
		2	10 ³	4/5	11
		2	10 ¹	3/5	10.5
	2 weeks	4	10 ¹	8/10	10.8
		2	10 ²	9/10	11
		1	10 ²	8/10	8.6
	3 weeks	4	10 ²	9/9	12.2
		2	10 ⁵	5/5	12.2
		2	10 ³	5/5	13.3
		2	10 ¹	3/5	13.7
		1	10 ¹	8/9	12.0
	4 weeks	4	10 ³	8/9	11.9
2		10 ²	8/9	11.6	
1		10 ¹	6/8	9.2	
5 weeks		4	10 ¹	6/8	11.2
Normal cells	—	4	10 ²	7/9	14.2
	—	2	—	0/4	—
	—	4	10 ²	8/8	11.2
	—	2	10 ²	3/4	9.6
	—	1	10 ²	4/5	9.2
	—	4	10 ⁵	8/8	10.2
Without cells	—	4	10 ¹	7/10	14.1
	—	—	10 ⁵	10/10	8.4
	—	—	10 ³	16/16	10.8
—	—	10 ¹	9/11	12.5	

* Donor spleen cells for adoptive immunization were obtained at weekly intervals after three i.p. weekly doses of 0.5 ml of 20% DV infected mouse brain suspension. The control group was given normal mouse spleen cells.

† Number of viable nucleated spleen cells given i.v. 24 h before DV was given i.c.

‡ LD₅₀ of DV per 0.03 ml of adult mouse brain given i.c.

§ D/T = Number of deaths/total number tested.

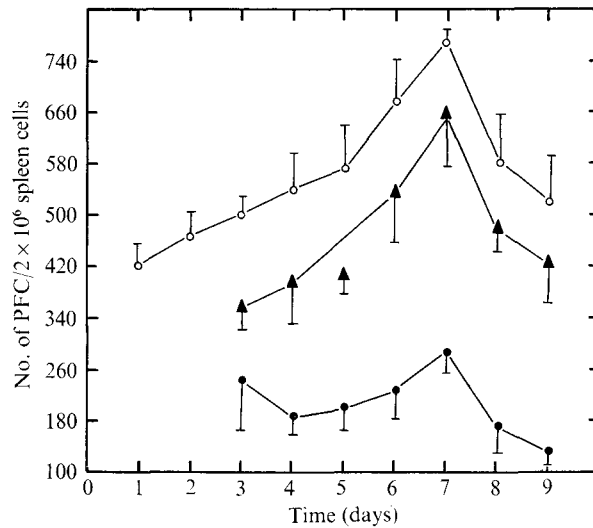


Fig. 2. Antibody plaque forming cells (PFC) per 2×10^6 spleen cells of mice on different days after i.p. inoculation of DV. Each point represents the mean value, with standard error of the mean, from multiple slides from 4 to 5 mice. ○—○, Mice given DV i.p.; ▲—▲, mice given DV i.p. followed 48 h later by 10^8 normal mouse spleen cells i.v.; ●—●, mice given DV i.p. followed 48 h later by 10^8 immune spleen cells i.v.

Effect of blockade of macrophages

The results summarized in Table 5 show that selective destruction of macrophages by silica had no effect on the outcome of the infection.

HI antibodies

The HI antibody titre at 1 and 2 weeks after immunization was 20 to 40 while at 3, 4 and 5 weeks, the titres ranged from 160 to 320. Sera of some of the mice given sensitized spleen cells i.v. showed antibody at a dilution of 1:20. HI antibodies were also noted in low titres in groups of mice given a single dose of DV i.p. or i.v. from the 10th or 12th day respectively.

DISCUSSION

Passive transfer of specific antiserum protected recipient mice against i.c. challenge with DV. Serum collected at early periods (1 to 2 weeks) after immunization provided minimum protection while serum collected at later periods (3 to 5 weeks) protected a greater number of mice with a higher dilution of serum. Poor protection by sera collected early may well be due to the lower concentration of antibody. In our experiments there was evidence of suppression of antigen-specific antibody secretion as detected by Jerne's plaque technique after transfer of sensitized spleen cells. The suppressor activity diminished at 4 to 5 weeks (see Table 3) which may be responsible for the higher concentration of HI antibody at this period and consequently greater protection by the serum transfer. Such suppression by transfer of sensitized cells was not due to feedback suppression of antibody formation by effector T-cells as suggested by Pang & Blanden (1976) because cells obtained 4 to 5 weeks after immunization were not able to significantly suppress the antibody forming cells in the spleen of recipient mice (see Table 3).

There are a number of host-arbovirus combinations in which virus replicates readily in

Table 2. *Adoptive immunization by serum obtained from specifically immunized mice protects against intracerebral challenge with DV*

Source of serum	Donor serum		Mortality		
	Period after immunization	Serum dilution	D/T†	Median day of deaths	% survival
Immune serum*	1 week	1:10	5/6	12.2	17
		1:100	5/6	13.4	17
		1:300	6/6	12.0	0
		1:500	6/6	11.5	0
	2 weeks	1:10	8/10	13.2	20
		1:100	4/6	10.8	33
		1:300	5/5	12.0	0
		1:500	5/5	12.8	0
	3 weeks	1:10	0/6	—	100
		1:100	1/4	16.0	75
		1:300	4/6	13.3	33
		1:500	5/5	14.0	0
	4 weeks	1:10	1/5	14.7	80
		1:100	1/5	18.0	80
		1:300	1/6	13.0	83
		1:500	4/6	17.2	33
	5 weeks	1:10	3/6	14.7	66
		1:100	0/6	—	100
		1:300	2/6	13.0	66
		1:500	6/6	14.0	0
Normal mouse serum‡	—	1:10	6/6	12.0	0
		1:100	6/6	12.2	0
Virus control	—	—	6/6	11.3	0

* Mice were immunized by three i.p. weekly doses of DV. Blood was collected at weekly intervals after the third dose and 0.5 ml of the serum dilution was injected i.p. into normal mice, followed 24 h later [by 10^2 LD₅₀ of DV i.c.

† D/T = Number of deaths/total number tested.

‡ In control mice, normal mouse serum was injected.

Table 3. *Suppression of antibody forming cells against DV in the spleen by adoptive transfer of sensitized spleen cells*

Donor cells* after immunization at different periods	AFC† (after DV challenge)	
	6th day	7th day
3 days	228 ± 52	283 ± 25
1 week	236 ± 27	322 ± 47
2 weeks	297 ± 54	324 ± 50
3 weeks	322 ± 62	330 ± 30
4 weeks	471 ± 25	634 ± 84
5 weeks	520 ± 50	757 ± 175

* Sensitized spleen cells obtained at different periods from mice given three doses of DV i.p. at weekly intervals, were transferred intravenously into recipient mice who were given DV i.p. 48 h earlier.

† Antibody forming cells (AFC) against DV were counted in the spleen of recipient mice on the 6th and 7th days after DV inoculation. Each value (the mean of triplicate tests on 4 to 5 mice ± s.d.) is expressed as AFC per 2×10^6 spleen cells.

Table 4. *Potential of DV infection by cyclophosphamide but not by anti-thymocyte serum*

Virus	Treatment*	Mortality	
		D/T†	Median day of death
DV	—	20/20	10·2
DV	ATS	17/20	12·2
—	ATS	0/10	—
DV	NRS	10/10	11·2
DV	CY	15/15	6·6
—	CY	0/10	—

* DV (10^2 LD₅₀) was injected intracerebrally and 0.1 ml antithymocyte serum (ATS) or normal rabbit serum (NRS) was given i.p. every other day from 2 days before infection to 12 days after infection. Cyclophosphamide (CY) was given in doses of 250 mg/kg 1 day after DV infection.

† D/T = Number of deaths/total number tested.

Table 5. *Failure of pre-treatment with silica to potentiate DV infection by peripheral routes**

Group	Treatment	DV challenge with 10^2 LD ₅₀	Mortality (D/T†)
A	50 mg silica i.p.	DV i.p.	0/12
B	50 mg silica i.p.	—	0/10
C	3 mg silica i.v.	DV i.v.	0/10
D	3 mg silica i.v.	—	0/8
E	—	DV i.p.	0/12
F	—	DV i.v.	0/15
G	—	DV i.c.	10/10

* Mice were pre-treated intravenously or intraperitoneally with 2 μ m sterile silica particles suspended in Hank's balanced salt solution to destroy the macrophages. DV was inoculated at 10^2 LD₅₀ by the same route 2 h later. The control mice were given either silica or virus alone.

† D/T = Number of deaths/total number tested.

brain but relatively poorly in peripheral tissues (Albrecht, 1968; Cole & Wisseman, 1969). In the present study and in an earlier study (Chaturvedi *et al.* 1974) high DV titres were noted in the brain tissue while liver, heart and spleen had low titres. Replication of DV associated with changes in enzymes and metabolites also occurs in skeletal muscles (Agrawal *et al.* 1978) and in liver and brain tissues (unpublished data) in mice. When inoculated by the intraperitoneal route, dengue virus fails to kill adult mice even with very large doses (Nathanson & Cole, 1970).

Out of four groups of mice sensitized with DV given in various doses by different routes, a significant LMI was noted only on two occasions, to the extent of 20 ± 1.8 and 21 ± 2.8 %. This is in contrast to the higher values of 60 to 65% LMI observed in Cocksackie B₄ virus infected mice with specific antigen (Chaturvedi *et al.* 1978). Stimulation of peripheral blood lymphocyte in DV infected monkeys have been noted in some experiments by Halstead *et al.* (1973).

Poor cell-mediated immunity in DV infection may be due to suppressor cell activity or to selective destruction of T-cells. This was shown to occur both in the circulation and in the thymus dependent areas of lymph nodes and spleen in dengue virus infection (Bhamaravati *et al.* 1967; Aung Khin *et al.* 1975).

Our results do not appear to demonstrate a role for cell-mediated control of DV infection in mice. There are three observations that support this assumption: (a) failure of adoptively transferred sensitized spleen cells to protect mice against challenge with a small dose

(10 LD₅₀) of DV; (b) failure of anti-thymocyte serum treatment of mice to potentiate DV infection; and (c) failure of adoptive transfer or reconstitution of immunosuppressed mice by sensitized spleen cells to protect against DV (Chaturvedi *et al.* 1977). Adoptive transfer of sensitized spleen cells could have provided protection by B-cells through elaboration of antibody as observed with Coxsackie B₄ virus (Chaturvedi *et al.* 1978). However, in the present experiments antibody against DV was detected only in low titres (1:20) in the sera of recipient mice on the 8th and 9th days after transfer of sensitized spleen cells. Such low titres of antibody were not sufficient to protect them against DV challenge.

We could not produce morbidity in mice after DV infection by i.p. or i.v. routes even after giving silica. If the resistance of mice to i.p. or i.v. infection with DV was due to macrophages then selective destruction of macrophages by silica should have caused increased susceptibility as noted in herpes simplex (Hirsch *et al.* 1970), yellow fever (Zisman *et al.* 1971) and Coxsackie B₃ virus (Rager-Zisman & Allison, 1973) infections. Therefore, macrophages do not appear to have a significant role in protection against DV. Our findings support the view of Van der Groen *et al.* (1977) that macrophages are not necessarily absolute barriers in arbovirus infections.

The findings of the present study substantiate our results reported elsewhere (Chaturvedi *et al.* 1977) that humoral antibody plays a critical role in recovery from acute dengue virus infection in mice whereas macrophages and cell-mediated immunity appear to have no protective role.

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