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Effect of Immunosuppression on Dengue Virus Infection in Mice

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SUMMARY

Mean survival time following intracerebral inoculation of dengue virus was reduced and the titre of the virus in the brain of immunosuppressed mice was markedly increased. A single dose of cyclophosphamide given 24 h after dengue virus i.c. or i.p. substantially reduced the number of antibody forming cells in the spleen. Three doses of dengue virus, each followed by cyclophosphamide 24 h later, produced specific hyporesponsiveness to the dengue virus but not to a heterologous virus (Coxsackie B4), with a reduction in antibody forming cells in the spleen of such animals against dengue virus but not against Coxsackie B4 virus. Adoptive immunity by antiserum was abolished along with increased titres of the virus in the brain of immunosuppressed mice but the protection could be restored by a second dose of antiserum. Pre-treatment of mice with immune or normal spleen cells i.v. or reconstitution of immunosuppressed mice by such cells had no effect. Thus, humoral antibodies play a crucially important role in host defence mechanism in recovery of mice from primary dengue virus infection.

INTRODUCTION

Immunosuppression has been used to convert a silent abortive virus infection into a lethal infection, accompanied by increased titres of the virus and an increased number of infected and destroyed cells in the target organs of various experimental models, namely, West Nile (Cole & Nathanson, 1968), encephalomyocarditis (Murphy & Glasgow, 1968), Sendai (Robinson, Cureton & Heath, 1969) and influenza (Hurd & Heath, 1975) viruses. A mouse-adapted strain of dengue virus produces acute lethal infection in adult mice if injected by the intracerebral (i.c.) route but is almost innocuous when given by other routes (Thind & Price, 1969; Tandon & Chaturvedi, 1977). Pre-treatment of mice with humoral antibody protects them against i.c. challenge with the dengue type 2 virus but immune splenic cells have no protective effect (Chaturvedi, Tandon & Mathur, 1977). Immunosuppression provides the means of assessing the role of immune response in recovery from primary virus infection. In the present study, therefore, the effect of immunosuppression by cyclophosphamide on the response of mice to dengue type 2 virus infection has been investigated with respect to active immunization and adoptive immunization by antisera or immune cells.

METHODS

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

Cyclophosphamide. The cyclophosphamide (CY) used was Endoxan-ASTA (Khandelwal Lab. Pvt. Ltd, ASTA Werke, A-G, West Germany). The dose of CY was 250 mg/kg body weight and was given by the intraperitoneal (i.p.) route.

Virus. Dengue type 2 (DV) virus (strain 23085), passed in the brain of mice, was originally obtained from the Virus Research Centre, Poona. The strain produced 100% mortality after intracerebral (i.c.) inoculation in infant and adult mice. The virus was used in the form of infected mouse brain suspension in doses of about 100 LD_{50}. The titres of virus were determined by i.c. inoculation in adult mice and the LD_{50} was calculated by the method of Reed & Muench (1938). Coxsackie B4 virus was obtained from the Haffkine Institute, Bombay and had undergone 12 passages in monkey kidney tissue culture (MKTC). The virus pool was prepared and the titre determined in MKTC.

Effect of CY on i.c. inoculation of DV. The effect of CY on the mortality pattern in mice was studied by giving the drug one day before, the same day and 1, 2, 3, 4, 5 or 6 days after i.c. DV inoculation. The mice were examined up to 21 days for illness and mortality. From the set of mice given CY at 1 day after DV, two mice were killed daily and the titre of virus was estimated in the brain tissue. For control, titres were determined daily in an equal number of mice given virus alone.

Effect of CY on i.p. inoculation of DV. CY was given one day before, the same day and one day after i.p. inoculation of DV. In control mice PBS was injected in place of CY and the mortality pattern was studied.

Effect of CY on adoptive immunization by antiserum. Mice were given CY i.p. followed 5 h later by 0·5 ml DV antiserum i.p., prepared in mice as described elsewhere (Chaturvedi *et al.* 1977). After 24 h the mice were challenged i.c. with DV. As controls, mice of one group (A) were given CY i.p. and normal mouse serum in place of antiserum, mice of control group B were given CY, antiserum and saline in place of virus and control group C received antiserum only. After this treatment control groups A and C were challenged i.c. with DV. The mortality pattern was recorded in each group (Table 1).

In a second experiment CY given i.p. was followed 5 h later by 0·5 ml of DV antiserum i.p. After 24 h these mice were challenged i.c. with DV, and 72 h later the same mice were again given 0·5 ml of DV antiserum i.p. Controls were included as in the first experiment and the mice were observed daily for 21 days. In both experiments virus titres in the brain of the two mice sacrificed daily were determined for 11 days after infection.

Effect of CY on adoptive immunization by cells. Spleen cells obtained 2 weeks after immunization of mice with 4 weekly i.p. doses of live DV were given i.v. to normal mice in doses ranging from 1×10^8 to 4×10^8 cells. After 24 h the mice were challenged i.c. with DV and 48 h later i.p. with CY. As a control, normal mouse spleen cells were used in place of immune cells (Table 2).

In a second set of experiments, mice were given DV i.c. followed by CY i.p. 24 h later, and immune spleen cells i.v. in varying doses (1×10^8 to 4×10^8 cells) after a further 48 h. Control mice were given normal mouse spleen cells (Table 3).

Effect of CY on antibody plaque forming cells. Mice were given 0·5 ml DV i.p. followed 24 h later by CY i.p. Control mice were given DV i.p. only. The experiment was repeated in a group of mice given virus by the i.c. route. Five to 7 mice were sacrificed daily for 11 days after infection. Spleens were collected aseptically and the cells were squeezed out with

Table 1. *Effect of CY on protection of mice by antiserum against DV challenge*

Group*	DV i.c.	CY i.p.	DV antiserum 1st dose	DV antiserum 2nd dose	Normal serum 1st dose	Normal serum 2nd dose	Saline i.p.	Mortality†
Expt. 1								
Test	+‡	+	+	−	−	−	−	20/20
Control A	+	+	−	−	+	−	−	14/14
Control B	−	+	−	−	−	−	+	0/15
Control C	+	−	+	−	−	−	−	0/12
Expt. 2								
Test	+	+	+	+	−	−	−	2/18
Control A	+	+	—	—	+	+	−	0/16
Control B	−	+	−	−	−	−	+	0/15

* A single dose of specific antiserum was sufficient to protect mice against subsequent challenge to DV 24 h later (Expt. 1, control C) but the protection was abolished by administration of CY (Expt. 1, Test). The protection by antiserum could be restored in such mice by a second dose of specific antiserum given 72 h after the first dose (Expt. 2, Test).

† Numerator, number dying; denominator, total number of mice.

‡ +, given; −, not given.

Table 2. *Effect of CY on mice pre-treated intravenously with immune or normal mouse spleen cells**

Group	No. of spleen (i.v.) cells	D.V. i.c.	CY i.p.	Mortality†
Immune cells	4×10^8	+‡	+	10/10
	2×10^8	+	+	9/10
	1×10^8	+	+	12/12
	4×10^8	+	−	8/8
Normal cells	4×10^8	+	+	12/12
	2×10^8	+	+	9/9
	1×10^8	+	+	12/12
	4×10^8	+	−	10/10

* Spleen cells, obtained 2 weeks after 4th weekly immunizing dose of DV, were given i.v. to normal mice which were challenged with DV i.c. 24 h later and CY i.p. 48 h later. As control, normal mouse spleen cells were given. Cell transfer had no effect on outcome of the infection.

† Numerator, number dying; denominator, total number of mice.

‡ +, given; −, not given.

forceps. The cells were washed in Hanks' basal salt solution and the viable nucleated cells were counted by the trypan blue dye exclusion test. The modified haemolytic plaque technique of Jerne, Nordin & Henry (1963) was used for counting antibody forming cells (PFC). Coating of the sheep RBCs with the virus (dengue or Coxsackie B4) was done by the technique of Russell, McCahon & Beare (1975) after treating RBCs with 5×10^{-4} M-KIO_4. The PFC assays were read after incubation of slides at 37 °C for 90 min in the presence of fresh guinea pig complement. The PFC were scored at ×30 magnification with a microscope. Only direct PFC were counted. From each mouse, multiple slides were prepared depending on the count of spleen cells.

Effect of CY on active immunization. Mice were immunized with live DV prepared from 20% (w/v) mouse brain suspension. DV (0·5 ml) was given i.p. on 0, 7 and 14 days (group A). In another group (B) of mice each injection of DV was followed by CY 24 h later (on days 1, 8 and 15). Similarly, a suspension prepared from normal mouse brain was given

Table 3. *Effect of reconstitution with immune or normal mouse spleen cells given DV and CY**

Group	DV i.c.	CY i.p.	No. of spleen cells (i.v.)	Mortality†
Immune cells	+‡	+	4×10^8	14/14
	+	+	2×10^8	12/12
	+	+	1×10^8	8/8
	+	−	4×10^8	12/12
Normal cells	+	+	4×10^8	10/10
	+	+	2×10^8	10/10
	+	+	1×10^8	10/10
	+	−	4×10^8	10/10

* Mice given DV i.c. followed 24 h later by CY were reconstituted with immune or normal mouse spleen cells (as in Table 2) 48 h later. Cell transfer had no effect on the outcome of the infection.
† Numerator, number dying; denominator, total number of mice.
‡ +, given; −, not given.

Table 4. *Effect of CY on response of actively immunized mice to challenge with homologous or heterologous virus**

Groups	Immunizing virus days 0, 7, 14	CY on days 1, 8, 15†	Challenge virus on day 25	Mortality‡
A	DV	−	DV	2/18
B	DV	+	DV	18/18
C	DV	−	Cox. B4	2/15
D	DV	+	Cox. B4	3/14
E	NMB§	−	DV	10/10
F	NMB	+	DV	12/12

* Mice given three doses of DV i.p., each followed 24 h later by CY, were challenged with homologous (DV) or heterologous (Coxsackie B4) virus and mortality was recorded. CY abolished the protective effect of active immunization against the homologous virus.
† +, drug given; −, drug not given.
‡ Numerator, number dying; denominator, total number of mice.
§ NMB = normal mice brain.

with (group F) or without CY (group E) to another set of mice as control. On the 25th day, mice were challenged with about 100 LD_{50} DV i.c. One set of these mice with (group D) or without (group C) treatment with CY were challenged with the 100 $TCID_{50}$ of Coxsackie B4 virus (Table 4). In one set of experiments the mortality of mice was recorded after challenge while in another set 3 to 4 mice were sacrificed daily after challenge and PFC were determined in the spleen as described above. In group B mice, the PFC against DV, and in mice of group D, the PFC against Coxsackie B4 virus, were studied. As control, PFC against Coxsackie B4 virus were studied in the spleen of normal mice after challenge with the same virus.

RESULTS

Effect on i.c. inoculation of virus

Fig. 1 shows the mortality in each group. All the mice given DV alone (control) died by the 12th day of virus inoculation while those given CY one day before, on the same day, or one day after the virus died after 5 to 6 days. This difference was statistically significant. In contrast CY did not significantly affect survival of mice when its administration was delayed by more than 1 day.

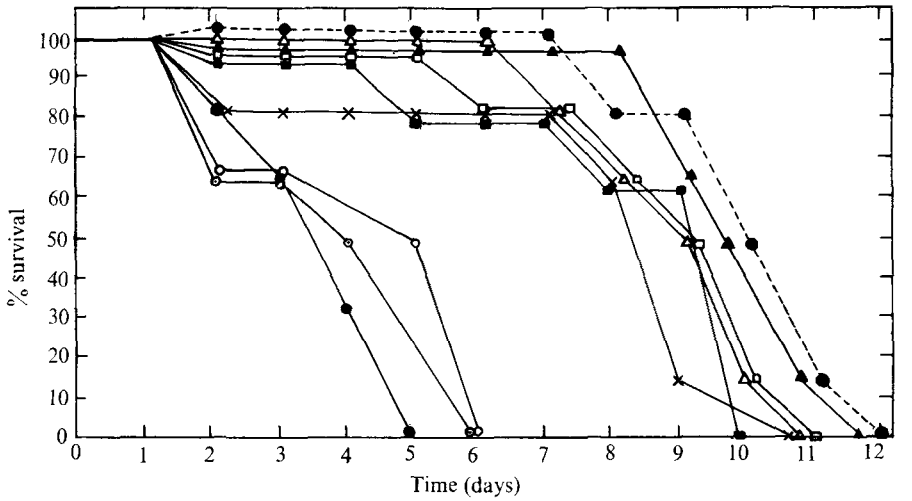

Fig. 1. Effect of CY on DV infection of adult mice. All mice received 100 LD_{50} i.c. of virus and CY at different periods before or after the virus. ●—●, 1 day before; ⊙—⊙, same day; ○—○, 1 day after; ×—×, 2 days after; △—△, 3 days after; ▲—▲, 4 days after; □—□, 5 days after; ■—■, 6 days after; ●----●, control. Each group consisted of 10 to 15 mice.

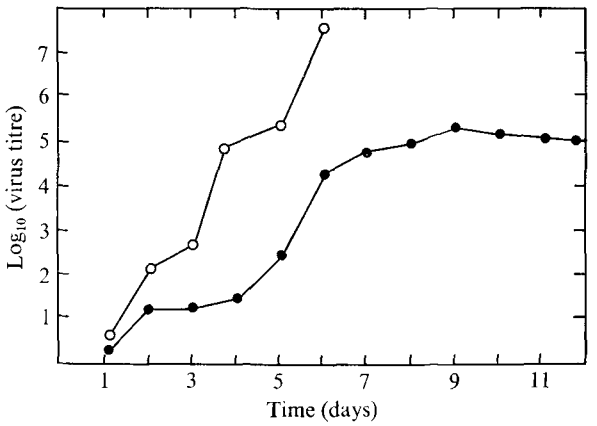

Fig. 2. Virus titres in the brain of infected mice on different days after inoculation. Each point represents an average for two mice. ●—●, Normal mice given DV and ○—○, mice given DV followed by CY 24 hours later.

The average titres of the virus in the brains of two mice at different days are shown in Fig. 2. It was noted that the titre of the virus was markedly higher from the 3rd day on in mice given CY. The maximum difference in titre in the CY treated and untreated mice was 2·7 logs on the 5th and 6th days.

No illness or mortality was noted in 20 mice given DV i.p. Similarly, no ill effects were seen in 30 mice given CY.

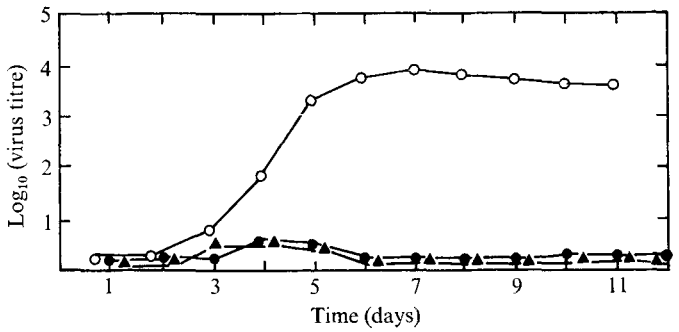

Fig. 3. Virus titres in the brain of infected mice on different days after inoculation. Each point represents an average for two mice. ○—○, Mice treated with antisera i.p. followed by CY 5 h later and DV i.c. 24 h later; ●—●, mice treated with antisera i.p. followed by D.V. i.c. 24 h later and ▲—▲, mice treated with antisera i.p. followed by CY 5 h later, DV i.c. 24 h later and antisera i.p. 72 h later.

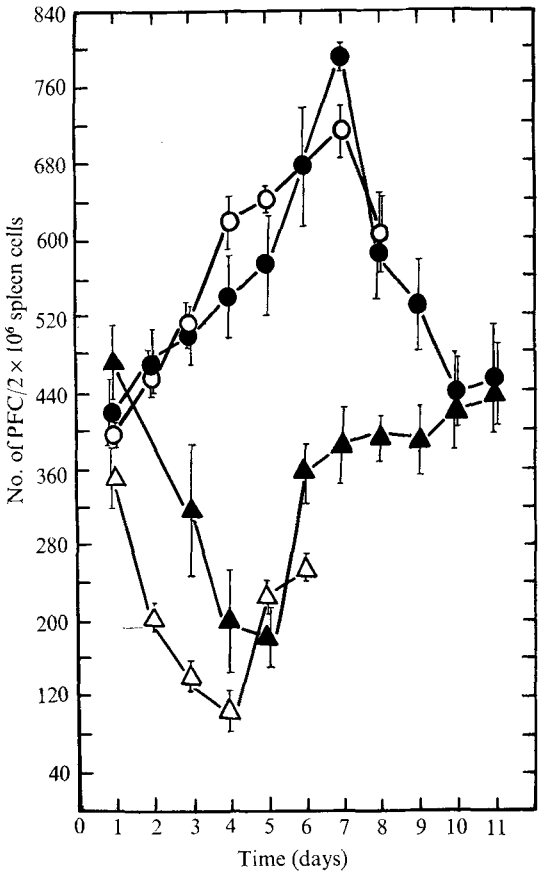

Fig. 4. Antibody plaque forming cells (PFC) in the spleen of infected mice on different days after inoculation. Each point represents the mean value from multiple slides from 5 to 7 mice with standard error of mean outlined. ○—○, Mice infected with DV i.c.; △—△, mice infected by DV i.c. followed by CY 24 h later; ●—●, mice infected with DV i.p. and ▲—▲, mice infected with DV i.p. followed by CY 24 h later.

Effect on adoptive immunization by antiserum

In the first experiment, mice receiving DV antiserum were protected against i.c. challenge with virus (control group C) while normal mouse serum provided no protection (group A). The antiserum failed to protect mice when CY was also given as all the 20 mice died (Table 1). In the second experiment where the second dose of antiserum was given 72 h later, 16 out of 18 mice survived the challenge with DV while controls behaved as in the first experiment (Table 1).

The titres of the virus in the brain of mice in different experiments have been shown in Fig. 3. It was noted that a small amount of virus was present in the brain between the 4th and 7th days and then disappeared in mice given virus and antiserum. In groups of mice also given CY the virus titres started increasing from the 3rd day but when two doses of antiserum were given a small amount of virus was detected for a few days.

Effect of CY on adoptive immunization by cells

The effect of CY on adoptive immunization by transfer of immune spleen cells has been summarized in Table 2. Pre-treatment of mice with immune or normal spleen cells did not affect the outcome of infection, nor did reconstitution with cells influence the effect of CY on mice receiving DV by i.c. inoculation (Table 3).

Effect on antibody forming cells

The effect of CY on antiviral PFC in the spleen of mice given a single injection of dengue virus by the i.c. or i.p. routes is shown in Fig. 4. Mice given virus alone by either route showed an increase in PFC which reached a peak on the 7th day. In contrast, the PFC decreased in CY treated mice, reaching the lowest count on the 4th or 5th day. The count increased later but the mice given virus i.c. and CY had died by the 6th day.

Effect on active immunization

Out of 18 immunized mice only 2 died after challenge with DV. In contrast mice given DV followed by CY showed no protection to challenge by DV as all the 18 mice became sick and died. All the control mice given normal mouse brain suspension died on challenge with DV irrespective of CY treatment (Table 4). CY treatment did not affect the results of challenge with Coxsackie B4 virus.

The immune response of the animals in this experiment was further investigated by studying PFC in the spleen (Fig. 5). Mice treated with three doses of DV, each followed by CY 24 h later (see group B, Table 4) showed negligible anti-DV PFC initially, on challenge with DV. The count gradually increased reaching 360 PFC/2×10^6 spleen cells on the 11th day. The response of similarly treated mice against a heterologous virus (Coxsackie B4) was studied to see whether the lowered PFC count was specifically for DV antigen. Results of this experiment (mice of group D, Table 4) showed that on challenge with Coxsackie B4 virus, PFC against Coxsackie B4 virus increased to 530 PFC/2×10^6 spleen cells on the 7th day and then declined. The untreated normal control mice on challenge with Coxsackie B4 virus showed a similar curve except that the number of PFC was much greater. Thus, treatment of mice with CY following an antigenic stimulus eliminates PFC against that antigen specifically but has little effect on PFC reactive against an unrelated antigen.

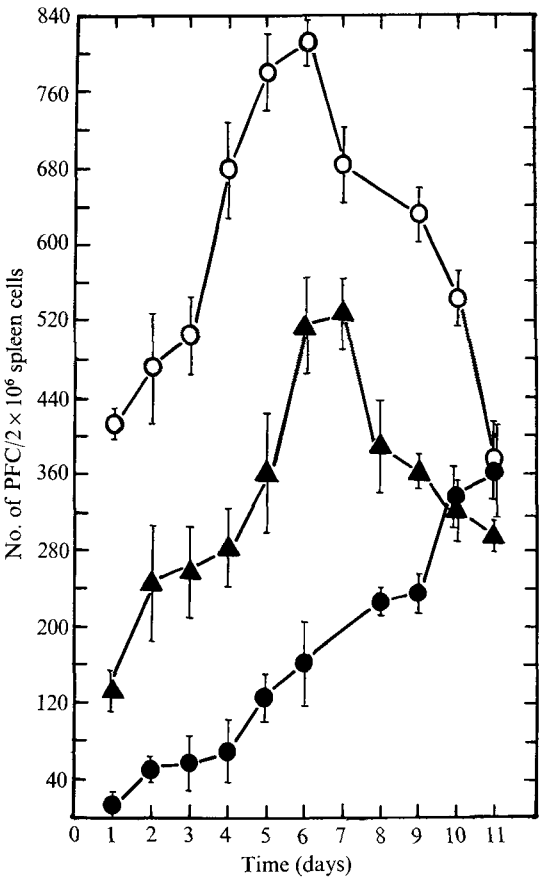

Fig. 5. Antibody plaque forming cells (PFC) in the spleen of mice given three weekly doses of DV, each being followed by CY 24 h later. ●—●, PFC against DV in mice challenged with DV i.p. on 25th day; ▲—▲, PFC against Coxsackie B4 virus in mice challenged with Coxsackie B4 virus i.p. on 25th day; ○—○, PFC against Coxsackie B4 virus in normal mice given Coxsackie B4 virus i.p. Each point represents the mean value of multiple slides from 3 to 4 mice with standard error of the mean outlined.

DISCUSSION

In spite of our repeated attempts, we have seen that DV given i.p. fails to produce sickness in mice while the same virus strain produces sickness and death on i.c. inoculation. Failure to produce sickness in mice by the i.p. route could be due to various reasons; either the DV fails to multiply sufficiently in the peritoneal tissue or the brain tissue is spared due to the blood-brain barrier when the virus is injected by i.p. route. However, the numbers of splenic PFC were nearly the same in both the situations (Tandon & Chaturvedi, 1977; Fig. 4). Therefore it appears that the extent of antigenaemia is also nearly the same in both situations.

The results of our experiments also showed that the mean survival time (MST) of infected mice was reduced when CY was given at the same time as virus. Similar reductions in MST have been reported with Coxsackie B3 virus (Rager-Zisman & Allison, 1973). In our experiments MST was found to be inversely related to the titre of virus in the brain tissues. Moreover, CY given i.p. was found to increase the virus titre of brain tissue in infected mice

(Fig. 2). Therefore it appears that CY helps the virus to multiply. It could be due to lowering of host-resistance (immunosuppression) to the infectious agent. It is known that CY causes latent damage to DNA (Santos & Owens, 1966). Therefore, it is possible that CY also affects the functional capacity of local mesenchymal tissue of the brain which in turn leads to rapid multiplication of the virus. Studies of histology and virus concentration in different organs of mice also showed significant findings in CY treated mice.

We had seen earlier that adoptive immunization of mice with DV antiserum protected them from subsequent i.c. challenge with DV (Chaturvedi *et al.* 1977). Therefore, antiserum does possess some protective role against DV infection. Either the antiserum neutralizes the infectivity of the virus or the immune-complexes are removed by the local phagocytic cells. However, CY treatment abolished the protection provided by the single dose of the antiserum (Table 1). It could be due to its effect on local glial cells which has been discussed earlier. A second dose of antiserum could reverse the immunosuppressive effects of CY, probably by neutralizing the virus in antibody excess in the presence of defective clearing of immune-complexes from the local area (Fig. 3). Pre-treatment or reconstitution of mice with normal or immune splenic cells did not affect the morbidity or mortality of mice given DV i.c. followed by CY i.p. This also supports our findings that cell mediated immunity plays no protective role in DV infection of mice (Chaturvedi *et al.* 1977).

The reduction in splenic PFC against DV was very marked in our experiments where 3 doses of DV were followed by CY treatment 24 h after each dose (Fig. 5). The results of this experiment could be explained on the basis of the effect of CY on rapidly proliferating cells. Therefore, the clonal proliferation and differentiation of antibody-forming cells (B lymphocytes) against DV might not have occurred, though the response to Coxsackie B4 virus remained almost unaffected. The study of PFC gives a good indication of antigen-specific hyporesponsiveness.

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