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Suppressor T cells for delayed-type hypersensitivity to Japanese encephalitis virus

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Summary. The delayed-type hypersensitivity (DTH) to Japanese encephalitis virus (JEV) and the suppressor cells controlling it and the antibody-forming cells in inbred Swiss mice have been studied. JEV induces DTH, with a peak response at day 7 following infection which persists at low levels at least up to 119 days. Suppressor activity appeared on day 18. It was transferable by immune spleen cells. Treatment of spleen cells with anti-Thy-1·2 antisera and complement abrogated the suppressor activity. The homogenate of the spleen was equally effective in mediating suppression of DTH and the humoral response as measured by direct antibody plaque-forming cell (IgM-PFC) assay. The suppressor activity was antigen-specific both on DTH and T helper for antibody response as the immune responses against SRBC or Coxsackie B_4 virus were not suppressed. The suppressor cells were sensitive to cyclophosphamide treatment when the drug was given 48 hr before their appearance. It is, therefore, concluded that in JEV infection of mice, antigen-specific suppressor T cells are generated, both for DTH and IgM antibody, which are cyclophosphamide-sensitive and mediate suppression through soluble product(s).

INTRODUCTION

Specific protection against virus infections is brought about by humoral or cell-mediated immune responses (CMI) or both. In Japanese encephalitis virus (JEV) infection, antibodies have been considered to play the key role in resistance to infection (Kobayashi & Oya, 1967; Mori, Kimoto & Takeya, 1970). The role of CMI in protection and pathogenesis of JEV infection is not well understood. Our studies have indicated the presence of short lived humoral and T cell-mediated protective immunity against JEV in mice (Mathur, Arora & Chaturvedi, 1983a). Further JEV infection in mice during pregnancy results in impaired delayed-type hypersensitivity (DTH) response in mothers and their offspring (Mathur, Arora & Chaturvedi, 1983b).

Suppressor cells are considered to be of central importance in the regulation of CMI and humoral immune responses. Suppressor cells are usually T lymphocytes (Morikawa *et al.*, 1977) but may be B lymphocytes (Katz, Parker & Turk, 1974) or macrophages (Lichtenstein *et al.*, 1981). We have described the generation of suppressor T cells in the spleen of JEV-primed mice which suppress JEV IgM antibody plaque-forming cells (PFC) in the spleen *in vitro* and *in vivo* (Mathur, Rawat & Chaturvedi, 1983c). In the present study we show the induction of DTH during JEV infection in mice and the generation of suppressor cells for it. The suppressor T cells for DTH and IgM PFC in the spleen are cyclophosphamide (CY)-sensitive and mediate suppression through soluble products.

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METHODS AND MATERIALS

Virus

Japanese encephalitis virus (JEV) strain 78668A was on the form of adult mouse brain suspension. Details

of the pathogenicity and growth of the virus in mice have been described earlier (Mathur, Arora & Chaturvedi, 1981; Mathur, Arora & Chaturvedi, 1982).

Animals

Inbred Swiss albino mice, 4–6 months of age were obtained from the mouse colony of this Department.

Treatment of mice with cyclophosphamide

Mice were injected intraperitoneally with 200 mg/kg body weight of cyclophosphamide (Endoxan-ASTA, Khandelwal Lab. Pvt. Ltd., ASTA Werke, A-G, West Germany) either 48 hr before or 7, 9 or 16 days after inoculation of antigen.

Preparation of spleen cells and splenic homogenate

A single cell suspension was prepared by teasing out spleen in chilled Eagle's minimum essential medium (MEM). Spleens were homogenized in chilled buffered saline (pH 7) using an MSE tissue homogenizer and then centrifuged in the cold at 3000 r.p.m. for 10 min. The clear supernatant was collected and stored in aliquots at $-70°$.

Spleen cell culture

A single cell suspension of normal spleen cells was made in MEM-HEPES supplemented with 10% foetal calf serum, 5×10^{-5} M 2-mercaptoethanol and antibiotics. Four millilitres of cell suspension (5×10^6 cells/ml) was dispensed in 5 cm glass petri-dishes and incubated at 37° in a humidified atmosphere of 5% CO_2.

Assay of antibody plaque-forming cells (PFC)

Suppressor activity of the spleen cells or the homogenate was studied by recording its affect on JEV-specific IgM antibody plaque-forming cells (PFC) in the spleen of mice by the technique of Jerne & Nordin (1963) as described earlier (Mathur *et al.*, 1983c). Spleen cell cultures were inoculated with 0·1 ml of 10^3 LD_{50} JEV at 0 hr, followed 24 hr later by inoculation of the primed spleen homogenate. PFC were recorded in cultures on days 3 and 4. The data have been presented after deducting background PFC and analysed by Student's *t*-test.

T cell depletion of spleen cells

The spleen cells suspension obtained 3 weeks after immunization, was treated with anti-Thy-1·2 antibodies (New England Nuclear) at 4° for 1 hr after which fresh guinea-pig serum (1:6 dilution) was added. After incubation at 37° for 1 hr the viable cells were counted by dye exclusion (Golub, 1971) as described earlier (Mathur *et al.*, 1983a).

Assay of DTH

Details of the procedure have been described earlier (Mathur *et al.*, 1983a). Briefly, mice were primed with 10% JEV-infected mouse brain suspension or SRBC (4×10^8). An eliciting dose of 0·025 ml of JEV (10^3 LD_{50}) or SRBC ($2{\cdot}5 \times 10^8$ SRBC in PBS) was injected subcutaneously (s.c.) in hind footpads. The footpad swelling was measured using a dial caliper (Oditest, M.C. Kroplin GMBH, Messenzengfabrik, West Germany) and the results were expressed as the percentage of footpad increase at 24 hr. The same volume of eliciting material was injected into the footpads of unimmunized control mice. Specific DTH represents readings after the substraction of background footpad increase induced by the eliciting antigen in normal mice.

RESULTS

The suppressor activity of the spleen cells and their homogenates obtained from JEV-primed mice has been assayed using two parameters: (i) the JEV-specific DTH as measured by 24 hr footpad swelling reaction, and (ii) the JEV-specific IgM PFC in the spleen cells.

DTH against JEV

Mice primed with JEV were challenged in the footpad at alternate days up to 21 days post-infection (p.i.) and

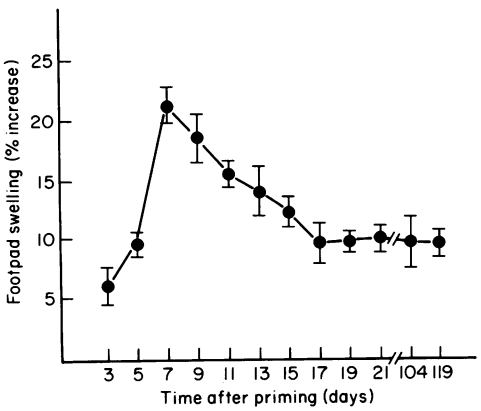

Figure 1. Effect on DTH response of JEV at different periods after priming. Five mice per group; mean ± SD.

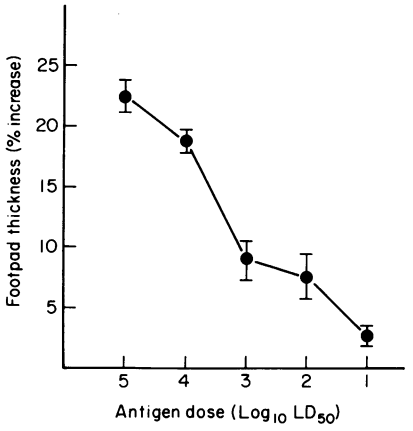

Figure 2. DTH to JEV in mice primed with different doses of virus. Groups of mice were injected with different dilutions of JEV. DTH was elicited 7 days later. Five mice were in per group. Each point represents mean ± SD.

then at 15 days intervals up to 17 weeks with the virus, and the increase on footpad thickness (FPR) measured 24 hr later. Mice developed DTH with a maximum response on the 7th day and a low level of DTH persisted for at least 119 days (Fig. 1). To study the dose–response of antigen on DTH, mice were primed with 10^1–10^5 LD_{50} of the virus, and the 24 hr increase in footpad thickness to challenge was measured on the 7th day. As shown in Fig. 2, progressive decrease in the FPR was observed with reducing dose of the virus.

Suppressor activity of spleen cells and their homogenate on DTH response

Spleens were collected at weekly intervals from 1 to 6 weeks after an injection of JEV i.p. and their suppressor effect on DTH was investigated. Groups of mice primed with JEV i.p. 48 hr earlier were given primed spleen cells or their homogenate i.v., and 24 hr FPR on day 7 was recorded. Findings summarized in Fig. 3 show a markedly suppressed DTH response by splenic cells as well as by their homogenate obtained at 3 and 4 weeks after priming. Control mice, which received normal mouse spleen cells or their homogenate, showed no suppressor activity at any period. To find out the precise day of appearance of the suppressor cells for DTH response, the suppressor activity of spleen homogenate obtained daily from day 16 to 20 after priming was studied. The data presented in Fig. 4a show a significant suppression of DTH response from day 18 onwards.

Suppressor activity of spleen cells and homogenate on antiviral plaque assay *in vitro*

We have observed earlier that spleen cells obtained at weekly intervals from JEV-infected mice on adoptive transfer suppressed PFC in spleen from 3 weeks (Mathur *et al.*, 1983c). To examine whether the suppressor activity was mediated through spleen homogenate also, spleens were collected from JEV-

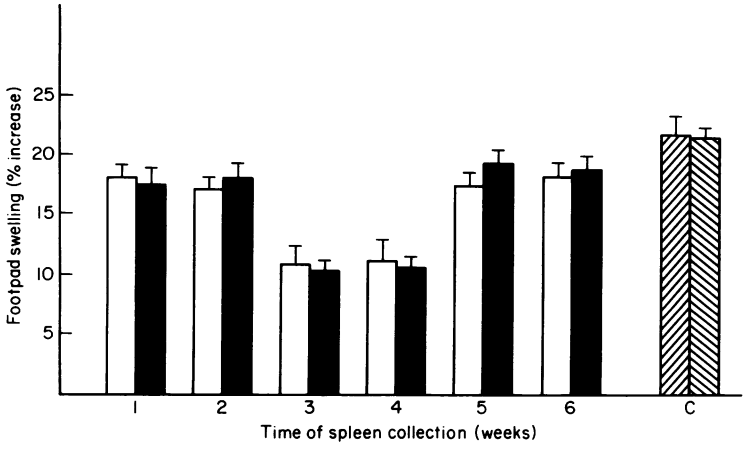

Figure 3. Development of DTH suppressor activity in the spleens of JEV primed mice. Mice were given JEV i.p. followed 48 hr later by i.v. inoculation with JEV-primed spleen cells (□), or primed spleen homogenate (■) collected at various times after priming. Control mice were given normal mouse spleen cells (■), or normal mouse spleen homogenate (▨). The 24 hr footpad reaction to JEV of recipient mice was measured at 7 days. Each column represent mean ± SD of five mice.

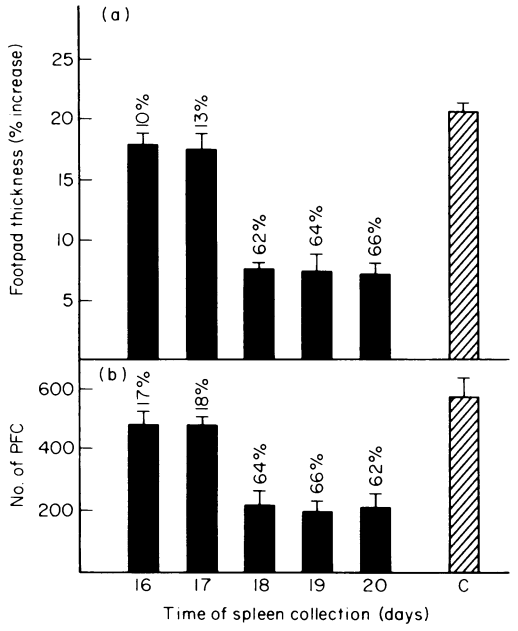

Figure 4. Suppressor activity of JEV-primed spleen homogenate collected on different days on PFC in spleen cell culture and DTH response in mice. (a) Mice were primed with JEV, followed 48 hr later by i.v. inoculation of JEV-primed spleen homogenate of days 16–20 (■); or inoculated with normal mouse spleen homogenate (▨). DTH was elicited 7 days later by measuring footpad swelling 24 hr after eliciting dose. (b) Normal mouse spleen cell cultures were inoculated with 10^3 LD_{50} JEV at 0 hr followed 24 hr later by inoculation of primed spleen homogenate (■); or cultures inoculated with normal spleen homogenate (▨). The cultures were harvested on days 3 and 4, and the IgM-PFC/2×10^6 cells against JEV were counted. Each observation represent the mean $\pm$ SD from multiple slides.

primed mice at every week p.i. and the homogenate were prepared. Figure 5 shows that the PFC count of primed spleen homogenate obtained after 1 and 2 weeks was $560 \pm 30/2 \times 10^6$ and $525 \pm 37/2 \times 10^6$ spleen cells respectively, showing negligible suppression, while significant suppressor activity was observed between 3 and 6 weeks (57–65% suppression). In order to investigate the day of appearance of suppressor activity, spleens were collected from JEV-primed mice from day 16 to 20. The suppressor activity was assayed *in vitro* inoculating spleen homogenate in JEV-stimulated cultures by estimating the PFC against JEV. The data presented in Fig. 4b show that on day 18 a significant suppression (64%) of PFC against JEV by spleen homogenate appeared. The homogenate of normal mouse spleen cells had negligible effect. Since maximum suppression was found during the 3rd week p.i. all further experiments were carried out on spleens collected at this period.

Correlation between number of spleen cells and DTH suppressor activity

Spleen cells obtained from mice at 3rd week after i.p. injection of JEV were transferred i.v. in different numbers into mice which were primed with JEV 48 hr before. Four days after, mice were challenged in footpad with 10^2 JEV and the increase in footpad thickness was measured 24 hr later. As shown in Fig. 6 there was a progressive increase in the per cent suppression, with an increasing number of spleen cells injected intravenously.

Abrogation of suppressor activity on DTH by T cell depletion

Primed spleen cells obtained at 3 weeks after infection with JEV were treated with anti-Thy-1.2 antibodies (New England Nuclear) and complement. The suppressor activity of these cells was screened *in vivo*. Group of mice were injected i.p. with JEV followed 48 hr later by i.v. inoculation of treated spleen cells (1×10^8). As a control, similarly treated normal mouse spleen cells and primed untreated spleen cells were inoculated. The DTH response was measured after 24 hr of eliciting dose of virus in the footpads. Findings presented in Table 1 show that pretreatment with anti-Thy-1.2 antibodies abrogates the suppressor activity of primed spleen cells of the 3rd week p.i.

Table 1. Effect of Thy-1.2 antiserum and complement treatment on the suppressor activity of DTH

Treatment of spleen cells	Specific 24 hr footpad increase (%)	Suppression (%)
Normal cells*	18·2	—
Immune cells†	5·2	71·5
Immune cells + ATS + C‡	16·4	10·0

Immune spleen cells were obtained at 3rd week after i.p. injection of JEV. Group of mice were inoculated with JEV i.p. followed 48 hr later by 1×10^8 spleen cells inoculation i.v. DTH was elicited 7 days after injection of antigen.

* Normal mouse spleen cells treated with diluent.

† Immune spleen cells treated with diluent.

‡ Immune spleen cells treated with anti-Thy-1.2 antiserum and complement.

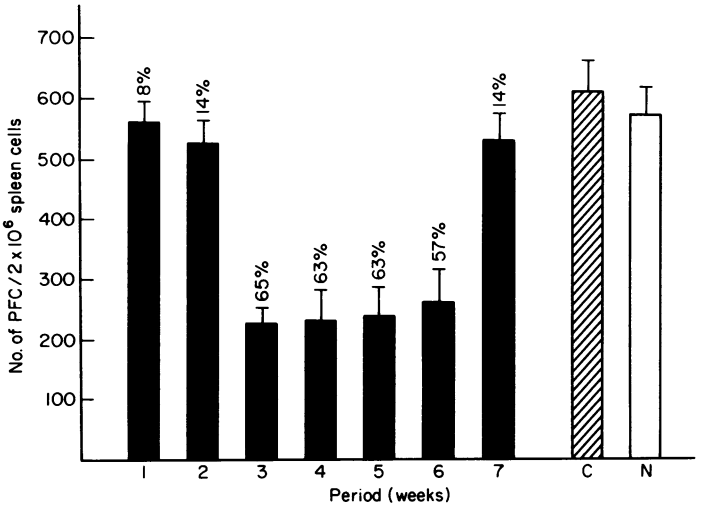

Figure 5. Suppressor activity of spleen homogenate at different periods after priming. Normal mouse spleen cell cultures were inoculated with 10^3 LD_{50} JEV at 0 hrs, followed 24 hr later by inoculation of primed spleen homogenate (■), cultures inoculated with diluent (■), culture inoculated with normal spleen homogenate (□). Each observation represent the mean ± SD from multiple slides of five mice.

Antigen specificity of suppressor activity on DTH response

Groups of mice primed with JEV or SRBC were injected 48 hr later with spleen cell homogenate i.v. obtained at 3 week after priming. Mice were challenged with respective antigens and the 24 hr footpad reaction to JEV of recipient mice was measured at day 7. As shown in Table 2, the DTH response against JEV was significantly suppressed, whereas there was no effect on the DTH to SRBC.

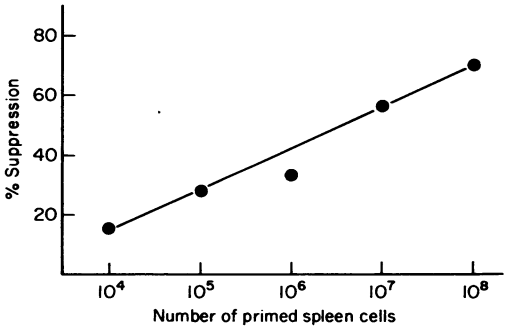

Figure 6. Correlation of the number of cells with the extent of suppression on DTH. Each point represent mean per cent suppression of footpad swelling observed in five mice.

Antigen specificity of suppressor activity on antibody plaque assay

Spleen cell cultures were stimulated by JEV, Coxsackie B_4 or SRBC, and the effect of JEV-primed spleen homogenate on their PFC count was examined. As shown in Table 2, the spleen homogenate suppressed 60% PFC against JEV, while suppression against the heterologous antigens was insignificant (6–10%).

Effect of cyclophosphamide on induction of suppressor cells

Groups of mice were injected with 200 mg/kg body weight of cyclophosphamide (CY) either 48 hr before or 7, 9 or 16 days after priming with JEV. The spleen cells were collected on 18 day p.i. and their suppressor activity was screened on DTH and IgM-PFC. It was observed that CY given on day 16 (48 hr before the appearance of suppressor activity) abrogated the suppressor activity on DTH as well as that on IgM-PFC (Fig. 7). CY given 2 days before or 7 or 9 days after priming with the virus had no effect on suppressor activity.

Table 2. Antigen specificity of suppressor activity of JVH

Stimulating antigen	Spleen homogenate	Footpad reaction*		PFC/2×10^6 spleen cells†	
		% increase	% suppression	No	% suppression
JEV					
Control	–	18·4	0	582 ± 25	0
Test	+	5·7	69·0	232 ± 13	60·0
SRBC					
Control	–	29·7	0	754 ± 20	0
Test	+	29·1	2·4	715 ± 23	6
Coxsackie B_4					
Control	–	ND	ND	509 ± 23	0
Test	+	ND	ND	460 ± 18	9·7

Immune spleen cells were obtained at 3rd week after i.p. injection of JEV.

* Mice were injected with antigen i.p. followed 48 hr later by inoculation of spleen homogenate i.v. DTH was elicited 7 days after injection of antigen.

† Spleen cell cultures were stimulated with antigen at 0 hr, followed 24 hr later with inoculation of spleen homogenate in test culture and diluent in control. On days 3 and 4 cultures were harvested and IgM-PFC against stimulating antigen were counted. Percent suppression was calculated from that of controls of the antigen.

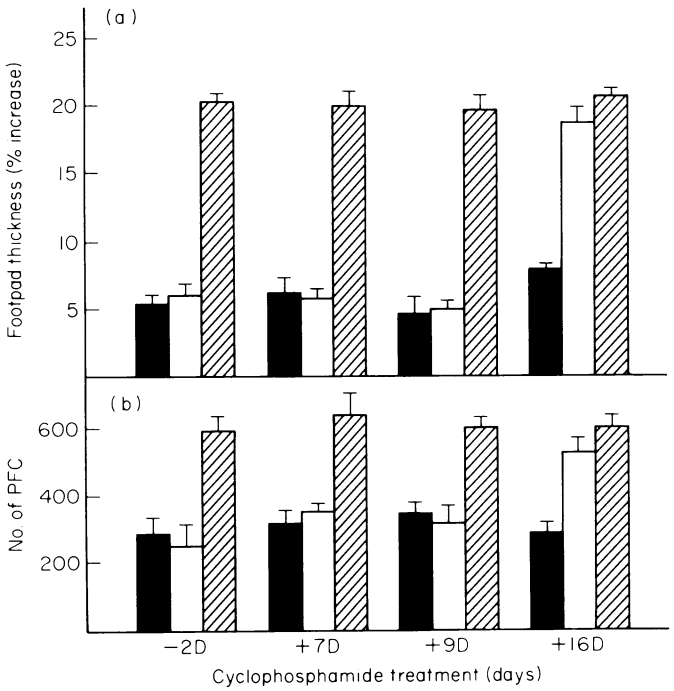

Figure 7. Effect of cyclophosphamide treatment on DTH and PFC to JEV. Mice were given CY i.p. 48 hr before or 7, 9 or 16 days after inoculation of JEV i.p. Spleens were collected at 18 days p.i. (a) Mice were primed with JEV, followed 48 hr later by i.v. inoculation of JEV-primed spleen homogenate (■); CY treated spleen homogenate (□); normal mouse spleen homogenate (▨). 24 hr footpad swelling reaction to JEV was measured of mice sensitized with JEV 7 days earlier. (b) JEV 10^3 LD_{50} inoculated into spleen cell culture at 0 hr, followed 24 hr later by inoculation of spleen homogenate from primed mice (■). CY-treated mice (□); normal mice (▨). Mean value + SD presented. Percent suppression was calculated from that of controls of antigen.

DISCUSSION

The data presented here show that JEV-primed spleen cells effectively suppress DTH and IgM-PFC against JEV. The suppressor activity of primed spleen cells on DTH is abolished by treating them with anti-Thy-1.2 antisera and complement, indicating thereby that they are T lymphocytes. Further, the homogenate of the primed spleen had an equally potent suppressor activity, therefore it appears that the suppressor T cells in our model mediated suppression through a soluble factor. In a number of experimental models, immunosuppression through soluble T cell products involving two or more steps has been demonstrated, viz. keyhole limpet haemocyanin (reviewed by Tada & Okumura, 1979), GAT (reviewed by Germain, 1980) and in dengue virus (DV) infection (Chaturvedi & Shukla, 1981). We have observed earlier the generation of a suppressor T cell in the spleen of JEV-primed mice which suppresses JEV IgM-PFC *in vitro* as well as *in vivo* (Mathur *et al.*, 1983c).

The findings demonstrate that induction of DTH against JEV is a dose-dependent phenomenon. The peak response appears at day 7 p.i., then it gradually declines but remains at a low level for at least 119 days. Suppressor T cells for DTH against JEV appear on the 18th day and last up to the 28th day after priming. In JEV-infected mice, the suppressor cells for antibody PFC in spleen are T lymphocytes, which suppress antiviral PFC on adoptive transfer in mice from the 3rd week onwards (Mathur *et al.*, 1983c). This supports our findings described here of the appearance of suppressor activity at day 18 by SF which lasts up to 6 weeks after priming. The late and sustained presence of T_s cells is also observed in influenza virus. The suppressor T cells for DTH against influenza virus appear 2 weeks after infection and are detectable in the spleen for at least 40 days thereafter (Liew & Russel, 1980).

Our earlier series of experiments show that the protection against virus challenge by adoptive transfer of serum or spleen cells can only be mediated when they are obtained within the first 2 weeks of immunization (Mathur *et al.*, 1983a). Consideration of these findings, together with the period of appearance of suppressor cells as observed earlier (Mathur *et al.*, 1983c) and in the present study, indicates a relationship between the two phenomena. The simultaneous appearance of suppressor T cells for both humoral and DTH response in JEV infection of mice is an interesting phenomenon. We have yet to investigate if the two functions are suppressed by the same set of suppressor cells, or if suppression is mediated by two different subpopulations of T lymphocytes. On the basis of the findings reported here, the indications are that they may be different, as one is short-lived while the other persists for 6 weeks.

Suppressor T cell activity for DTH and humoral responses was abrogated when CY was inoculated on day 16 p.i. (48 hr before the appearance of suppressor cells), while CY had no effect on suppression when given 48 hr before or 7 or 9 days after the virus inoculation. The effect of CY on immune functions depends upon the time course relationship with the antigen. Shand & Liew (1980) have reported that CY, if given shortly prior to SRBC, suppresses antibody formation, but when given 48 hr before the antigen, even in smaller doses, it eliminates precursors of the suppressor T cells, thus removing control on the immune response. The findings of the present study may appear paradoxical in that CY given 16 days after the antigen inhibits generation of suppressor cells, but a closer study of the findings revealed that they appear on the 18th day and the precursor cells are exposed to CY before mature suppressor cells are formed. The delayed appearance of suppressor cells may be due to their genesis through stimulation by antigen–antibody immune complexes (Taylor & Basten, 1976). Further studies are needed to elucidate this point.

The suppressor T cell activity was antigen-specific, as DTH against SRBC and IgM-PFC against Coxsackie B_4 virus and SRBC were not suppressed. Previously we have reported suppression of IgM-PFC against dengue virus (DV) by these suppressor cells (Mathur *et al.*, 1983c). Both JEV and DV belong to the Flavi group of Toga viruses and produce HAI antibodies in man and animals which cross-react with the antigens of the two viruses as well as with those of other viruses belonging to the same group. This cross-reaction appears to be expressed on the suppressor cells, also resulting in suppression of IgM PFC against DV. On the other hand, the suppressor signal generated in mice by DV is absolutely antigen-specific throughout the suppressor pathway, involving several populations of T lymphocytes (Chaturvedi & Shukla, 1981). Antigen-specific suppressor T cells have also been shown for DTH in influenza (Liew & Russel, 1980) and reoviruses (Greene & Weiner, 1980).

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REFERENCES

CHATURVEDI U.C. & SHUKLA M.I. (1981) Characterization of the suppressor factor produced in the spleen of dengue virus-infected mice. *Ann. Immunol. (Pasteur Inst.)*, **132C**, 245.

GERMAIN R.N. (1980) Antigen-specific T cell suppressor factors: mode of action. In: *Lymphokine Reports* (eds. E. Pick and M. Landy), Vol. 1, p. 7. Academic Press, London.

GOLUB E.S. (1971) Brain associated theta antigen: reactivity of rabbit anti-mouse brain serum with mouse lymphoid cells. *Cell. Immunol.* **2**, 353.

GREENE M.I. & WEINER H.I. (1980) Delayed hypersensitivity in mice infected with reovirus. II. Induction of tolerance and suppressor T cells to viral specific gene products. *J. Immunol.* **125**, 283.

JERNE N.K. & NORDIN A.A. (1963) Plaque formation in agar by single antibody producing cells. *Science*, **140**, 405.

KATZ S.I., PARKER D. & TURK J.L. (1974) B cell suppression of delayed hypersensitivity reactions. *Nature (Lond.)*, **251**, 550.

KOBAYASHI M. & OYA A. (1967) Presented at the 16th Annual Meeting of Japanese Virologists.

LICHTENSTEIN A., MURAHATA R., TERPENNING M., CANTRELL J. & ZIGHELBOIN J. (1981) Activation and mechanism of action of suppressor macrophages. *Cell. Immunol.* **64**, 150.

LIEW F.Y. & RUSSELL S.M. (1980) Delayed-type hypersensitivity to influenza virus: Induction of antigen specific suppressor T cells for delayed type hypersensitivity to haemagglutinin during influenza virus infection in mice. *J. exp. Med.* **151**, 799.

MATHUR A., ARORA K.L. & CHATURVEDI U.C. (1981) Congenital infection of mice with Japanese encephalitis virus. *Infect. Immun.* **34**, 26.

MATHUR A., ARORA K.L. & CHATURVEDI U.C. (1982) Transplacental Japanese encephalitis virus (JEV) infection in mice during consecutive pregnancies. *J. gen. Virol.* **59**, 213.

MATHUR A., ARORA K.L. & CHATURVEDI U.C. (1983a) Host defence mechanisms against Japanese encephalitis virus-infection in mice. *J. gen. Virol.* **64**, 805.

MATHUR A., ARORA K.L. & CHATURVEDI U.C. (1983b) Immune response in congenitally Japanese encephalitis virus-infected baby mice and their mothers. *J. gen. Virol.* **64**, 2027.

MATHUR A., RAWAT S. & CHATURVEDI U.C. (1983c) Induction of suppressor cells in Japanese encephalitis virus infected mice. *Br. J. exp. Path.* **64**, 336.

MORIKAWA S., BABA M., HARADA T. & MITSUOKA A. (1977) Studies on delayed hypersensitivity in mice. III. Evidence for suppressive regulatory T cell population in delayed hypersensitivity. *J. exp. Med.* **145**, 237.

MORI R., KIMOTO K. & TAKEYA K. (1970) The role of thymus in antibody production and in resistance to Japanese encephalitis virus infection. *Archiv ges. Virusforsch.* **29**, 32.

SHAND F.L. & LIEW F.Y. (1980) Differential sensitivity to cyclophosphamide of helper T cells for humoral responses and suppressor T cells for delayed-type hypersensitivity. *Eur. J. Immunol.* **10**, 480.

TADA T. & OKUMURA K. (1979) The role of antigen-specific T cell factors in the immune responses. *Adv. Immunol.* **28**, 1.

TAYLOR R.B. & BASTEN A. (1976) Suppressor cells in humoral immunity and tolerance. *Br. med. Bull.* **32**, 152.