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INDUCTION OF SUPPRESSOR CELLS IN JAPANESE ENCEPHALITIS VIRUS INFECTED MICE

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Summary.—Adoptive transfer of spleen cells obtained from mice primed with Japanese encephalitis virus (JEV) suppressed IgM antibody plaque forming cells (PFC) against JEV in the spleen. Similar suppression of PFC was also shown *in vitro* by adding primed spleen cells to JEV-stimulated spleen cell cultures. The suppressor activity appeared sharply in the third week after priming and persisted up to 6 weeks. By using various cell separation procedures it was found that the suppressor activity resided in the T cell enriched fraction and not in B cells or macrophages. Sensitivity of the cells to treatment with anti-Thy 1·2 antiserum and complement confirmed that suppressor cells were T lymphocytes. It was noted that the suppression was effective against dengue virus antigen also. Our findings thus show generation of suppressor T lymphocytes in JEV-infected mice.

PERSISTENT JAPANESE encephalitis virus (JEV) infection has been observed in female mice given virus i.p. during pregnancy in spite of the production of antibodies, and has been shown to be reactivated during a subsequent pregnancy (Mathur, Arora and Chaturvedi, 1982*a*). Virus infection that becomes persistent is often associated with immunosuppression in the host (Notkins, Mergenhagen and Howard, 1970). We have reported the transient presence of cell mediated immunity (CMI) and IgM antibodies up to 2 weeks after JEV priming; both protected recipient mice against JEV challenge on adoptive transfer (Mathur, Arora and Chaturvedi, 1983*a*). We have observed earlier that the inoculation of JEV i.p. in pregnant mice leads to transplacental transmission of virus to foetuses (Mathur, Arora and Chaturvedi, 1981). Further studies demonstrated depressed cell-mediated immune responses to specific (JEV) and heterologous (SRBC) antigens in JEV infected mice (mothers) and their congenitally infected baby mice (Mathur, Arora and Chaturvedi, 1983*b*). Persistence of JEV in mice, the transient presence of protective antibodies and CMI, and depressed CMI responses in congenitally infected infants and their mothers lead us to further study of the genesis of these phenomena.

The immune response to an antigen is controlled by 2 sets of T lymphocytes, the helper T cells and the suppressor T cells; the latter may act via the helper T cells or directly on the effector T or B cells. The suppressor cells have been shown to play a key role in regulating the immune response, therefore the present study was designed to look for suppressor cells in JEV-primed mice. The findings reported here indicate generation of suppressor T

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lymphocytes in the spleens of JEV-primed mice which mediate virus group-specific immunosuppression.

MATERIALS AND METHODS

Animals.—Four to six months old male inbred Swiss albino mice obtained from the mouse colony of this department were used.

Virus.—Japanese encephalitis virus (JEV) strain 78668A was adapted to grow intracerebrally (i.c.) in adult Swiss albino mice and used as infected mouse brain suspension. Details of the virus have been given earlier (Mathur *et al.*, 1982*b*). Another antigen used in this study was dengue type 2 virus (DV) strain 23085 (Chaturvedi, Tandon and Mathur, 1977). Mice were primed with a single dose of 0·5 ml of 20% JEV infected mouse brain suspension intraperitoneally (i.p.); this route of injection produced no clinically evident disease. Control animals were inoculated with the same dose of normal mouse brain suspension.

Preparation of spleen cells.—The spleens were collected aseptically and teased out in chilled Eagle's minimum essential medium (MEM) containing 10% foetal calf serum (FCS). A single cell suspension was prepared. In different preparations 90–95% of cells were viable as determined by the trypan blue exclusion test.

Culture of spleen cells.—The spleen cells were cultured in 5 cm glass Petri dishes in 4 ml (5 10^6 cells/ml) MEM-HEPES containing 10% FCS, 5×10^{-5}M 2-mercaptoethanol (2-ME) and antibiotics. The cultures were incubated at 37° in the presence of 5% CO_2.

Preparation of enriched spleen cell population.—The spleen cell suspension obtained 3 weeks after immunization was fractionated to obtain enriched spleen cell populations. Macrophages were depleted by treating spleen cell suspensions with carbonyl iron (Lymphocyte Separator Reagent, Technicon Instruments, N.Y., U.S.A.). From the macrophage-depleted spleen cells T and B lymphocyte-enriched populations were obtained by filtration through a nylon wool column by the technique of Julius, Simpson and Herzenberg (1973). The glass-adherent cells were separated from glass-non-adherent cells by layering spleen cells in glass Petri dishes and incubating for 2 h at 37° in the presence of CO_2. Glass-non-adherent cells were collected by decanting the fluid gently and washing the glass-adherent cells. These latter were removed with the help of a rubber-tipped policeman. More than 88% of glass-adherent cells were phagocytic and have been considered to be macrophages.

Experimental design.—The spleens of JEV-primed mice were examined at weekly intervals from 1–8 weeks for the presence of suppressor cells. The indicator system for the suppressor activity was plaque forming cells (PFC) producing JEV-specific IgM antibody in the spleen of mice. The PFC were studied by the haemolysis-in-gel technique of Jerne and Nordin (1963) as described in detail earlier (Mathur *et al.*, 1983*a*). The suppressor activity was assayed *in vivo* and *in vitro*. For *in vivo* assay, mice were given 10^3 LD_{50} JEV i.p. followed 48 h later by the test preparation of primed spleen cells i.v. JEV specific IgM PFC were counted in the spleens of the mice on days 5 and 6. For *in vitro* assay spleen cell cultures were inoculated with 10^3 LD_{50} JEV at 0 h followed 24 h later by the addition of primed spleen cells. JEV specific IgM PFC were counted in cultures on days 3 and 4. We have observed that maximum JEV IgM PFC were produced in the spleen on days 5 and 6 *in vivo* (Mathur *et al.*, 1983*a*) and on days 3 and 4 *in vitro*, therefore, in the present study the findings were recorded on these days. From each mouse or spleen culture multiple slides were prepared. For the PFC counts the suppressor activity of spleen cells obtained from 3–5 mice at each time interval was studied. The data have been presented after deducting background PFC; and the effect of primed spleen cells has been expressed as percent suppression of PFC as compared with those which received only the virus.

The data have been subjected to Student's t test for statistical evaluation. P values of less than 0·05 were considered significant.

RESULTS

Dose response of the primed spleen cells

The relationship between the number of primed spleen cells and the suppression of JEV specific PFC was studied. The dose response curve shown in Fig. 1 indicates a linear relationship. In the present study 5×10^6 splenic cells were used throughout the study to screen the suppressor activity of the primed cells.

Suppressor activity at different periods after priming

The suppressor activity of primed spleen cells obtained 1–8 weeks after a single JEV i.p. inoculation was screened in spleen cell cultures. The findings presented in Fig. 2 show that the primed spleen cells obtained

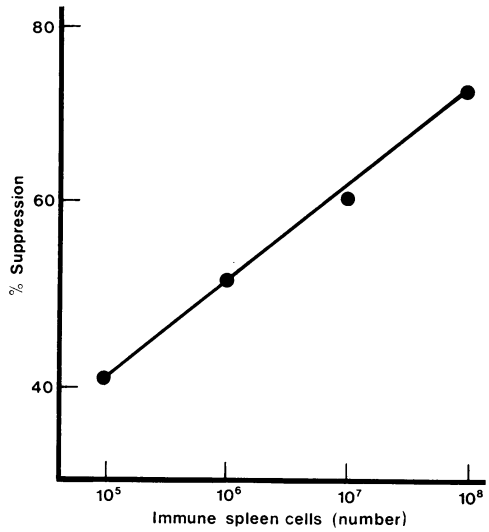

FIG. 1.—Suppressor activity of JEV-primed spleen cells *in vitro*. Correlation of the number of cells transferred with the extent of suppression.

at 1 and 2 weeks had little effect on the PFC (suppression 16%), while a significant suppression of IgM PFC was caused by primed cells obtained at 3, 4, 5 and 6 weeks (64, 61, 59 and 56% respectively). At later periods the suppressor activity diminished. The PFC count in cultures given the virus alone or virus and normal spleen cells was similar.

The suppressor activity was also screened *in vivo* in mice. As shown in Fig. 3 the PFC count in mice given primed spleen cells collected one week after priming was $604 \pm 39{\cdot}3/2 \times 10^6$ spleen cells and in mice given normal cells was $730 \pm 63/2 \times 10^6$, showing little suppression (17%). Similar activity (18%) was obtained with cells of 2nd week. Marked suppressor activity was observed when primed cells obtained after 3 and 4 weeks were given (49 and 46% respectively). Since *in vitro* and *in vivo* findings were similar for cells collected 3 and 4 weeks after priming, the suppressor activity of cells collected after longer intervals was screened *in vitro* only. Spleen cells obtained from normal mice had no effect on PFC.

Suppressor activity of enriched spleen cell subpopulations

In order to delineate the cell type responsible for suppressor activity in JEV primed mice, splenic cells separated by various techniques were screened. In the previous experiments maximum suppressor activity was seen 3 weeks after priming, therefore, in this experiment the enriched cells were obtained from spleens 3 weeks after priming. The suppressor activity of cell populations was studied *in vitro* using spleen cell cultures. The findings presented in Fig. 4 show the effect of macrophage-depleted splenic cells on PFC. It was observed that such cells suppressed 66% of PFC against JEV while control cells produced no suppression. The data presented in Fig. 5 show that the primed T cell enriched subpopulation suppressed 55% PFC while no suppression was observed with the enriched normal mouse T cells or with unseparated mouse spleen cells. Primed or normal B lymphocytes had negligible suppressor activity. The findings summarized in Fig. 6 show that glass-nonadhering cells suppressed 64% PFC. Negligible suppression was noted with glass-adherent primed cells.

T cell depletion reduces suppressor activity

The findings of the above experiments indicate that the suppressor activity of the primed spleen cells is mediated by the T lymphocyte enriched subpopulation. To obtain further support for this conclusion the following experiment was done. Primed spleen cells obtained 3 week after priming were treated with monoclonal antibody to Thy 1.2 (New England Nuclears) and complement by the technique of Golub (1971) as described earlier (Mathur *et al.*, 1983*a*). The suppressor activity of these cells was screened *in vitro*. For control similarly treated normal mouse spleen cells were included. Findings presented in the table show that untreated primed spleen cells suppressed JEV-specific PFC by 58% while the cells treated with anti-Thy 1.2 antibodies

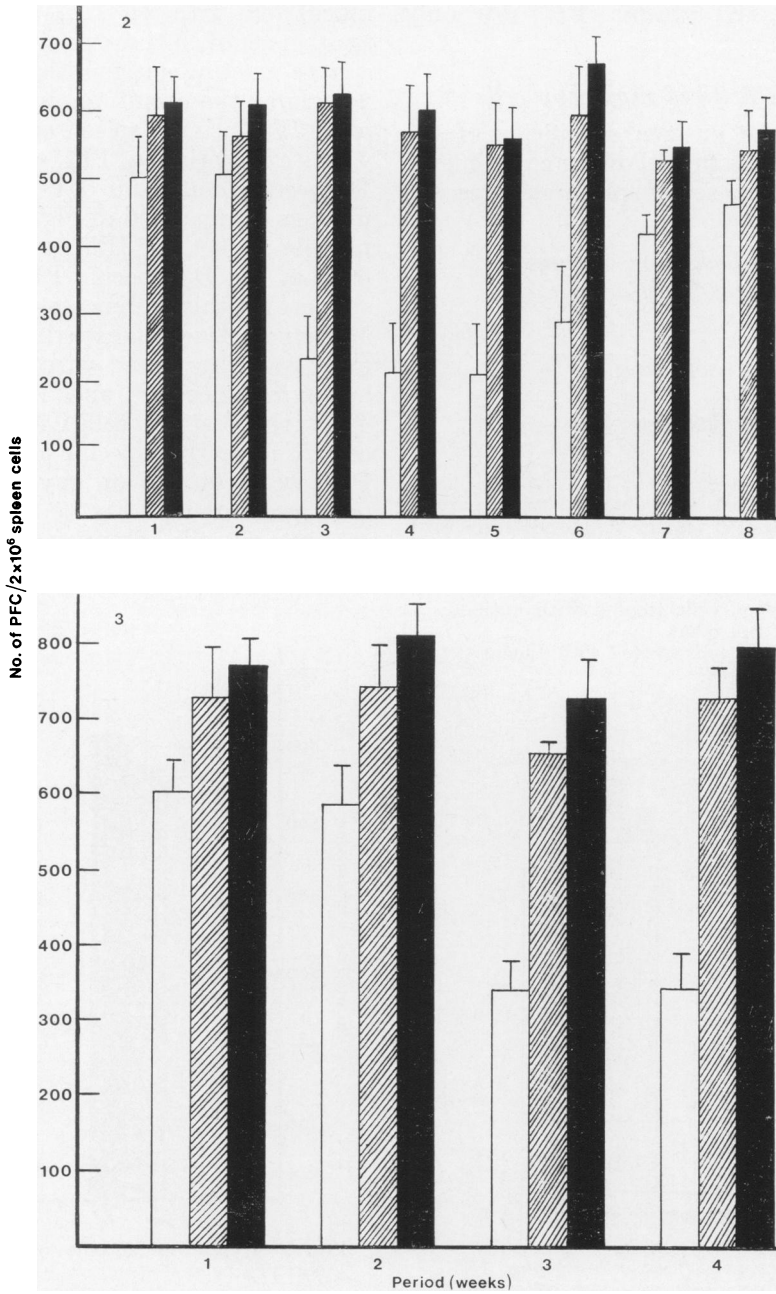

FIG. 2.—Suppressor activity of spleen cells *in vitro*, at different periods after priming. Normal mouse spleen cell cultures were inoculated with JEV 10^3 LD_{50} at 0 h followed 24 h later by inoculation of 5×10^6 primed spleen cells. Open columns, cultures inoculated with primed spleen cells; hatched column, cultures inoculated with normal spleen cells; filled columns, cultures with diluent. The cultures were harvested on days 3 and 4 and IgM PFC against JEV were counted.

FIG. 3.—Suppressor activity of spleen cells *in vivo* at different periods after priming. Mice given JEV i.p. followed 48 h later by 10^8 primed cells i.v. Open columns, mice given 10^8 primed spleen cells i.v.; hatched columns, mice given 10^8 normal spleen cells i.v.; filled columns, mice given diluent i.v. On days 5 and 6 after JEV the mice were killed and spleens were taken out and IgM PFC against JEV were counted.

suppressed JEV-specific PFC by only 10%.

Antigen specificity of suppressor cells

To test the antigen specificity of the suppressor cells the following experiments were done. One set of spleen cultures was inoculated with 10^3 LD_{50} of JEV and another set of cultures was inoculated with a cross-reacting antigen, the dengue virus. 24 h later these cultures were inoculated with JEV primed spleen cells obtained 3 weeks after priming. PFC against JEV or DV were counted on days 3 and 4. The findings summarized in Fig. 7 show a suppression of 64%, of JEV specific PFC, and 60% of the DV-specific PFC. Additional support for this finding was obtained by a Mackaness type of experiment in which mice were inoculated with JEV and DV i.p. simultaneously, and 48 h later with JEV primed spleen cells i.v. In the spleen cells of mice JEV specific and DV specific PFC were counted on day 5 and 6. The data presented in Fig. 7 show that the suppressor cells suppressed 58% JEV-specific PFC and 55% DV-specific PFC.

TABLE.—*Reduction of suppressor activity by depletion of T cells*

Cells added	PFC/(2×10^6 cells) No.	% suppression
Treated primed cells*	491 ± 46	10
Untreated primed cells†	231 ± 62	58
Normal cells	549 ± 60	0
None	547 ± 63	0

Primed spleen cells were obtained 3 weeks after a single i.p. dose of 0·5 ml of 20% JEV suspension. Normal mouse spleen cell cultures inoculated with JEV 10^2 LD_{50} at 0 h followed 24 h later by 5×10^6 spleen cells.

* Primed spleen cells treated with antibody to Thy 1·2 and complement.

† Primed spleen cells treated with diluent.

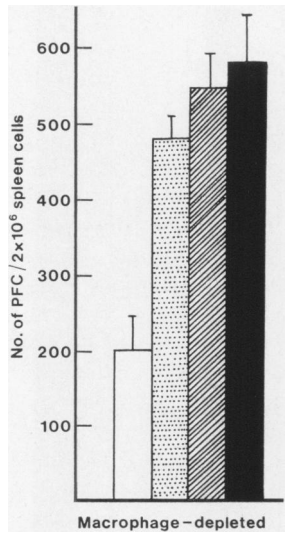

FIG. 4.—Suppressor activity of macrophage-depleted primed spleen cells *in vitro*. JEV 10^3 LD_{50} inoculated in spleen cell culture at 0 h followed 24 h later by inoculation of 5×10^6 primed macrophage-depleted cells (open column); inoculated with normal macrophage-depleted cells (stippled column); inoculated with normal total spleen cells (hatched column); inoculated with diluent (filled column) The cultures were harvested on days 3 and 4 and IgM PFC against JEV were counted.

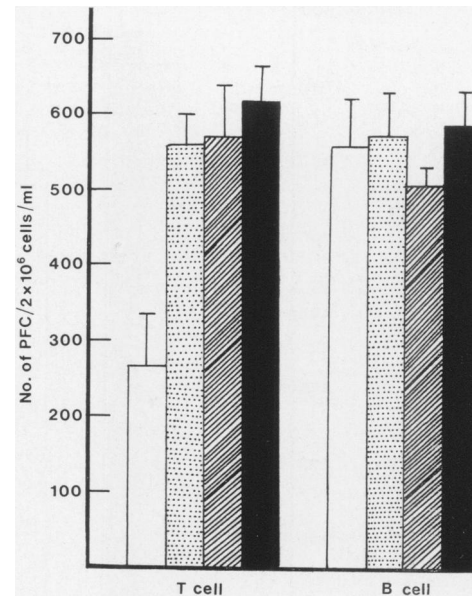

FIG. 5.—Suppressor activity of T or B cell enriched primed spleen cells *in vitro*. JEV 10^3 LD_{50} inoculated in spleen cell culture at 0 h followed 24 h later by inoculation of 5×10^6 primed macrophage-depleted cells (open column); inoculated with normal macrophage-depleted cells (stippled column); inoculated with normal total spleen cells (hatched column); inoculated with diluent (filled column). The cultures were harvested on days 3 and 4 and IgM PFC against JEV were counted.

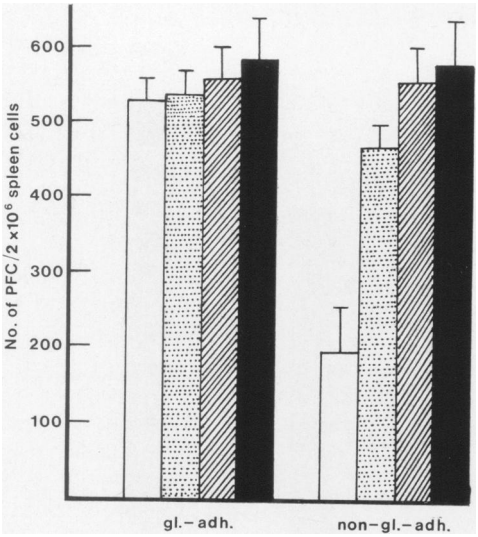

FIG. 6.—Suppressor activity of glass-adherent or non-glass-adherent cells. JEV 10^3 LD_{50} inoculated in spleen cell culture at 0 h followed 24 h later by inoculation of 5×10^6 primed macrophage-depleted cells (open column); inoculated with normal macrophage-depleted cells (stippled column); inoculated with normal total spleen cells (hatched column); inoculated with diluent (filled column). The cultures were harvested on days 3 and 4 and IgM PFC against JEV were counted.

DISCUSSION

Our study has demonstrated development of suppressor cells in the spleen of JEV primed mice which suppress IgM PFC against JEV on adoptive transfer in mice (*in vivo*) and also when added to JEV stimulated spleen cell cultures (*in vitro*). It was a dose dependent phenomenon, with greater suppression of PFC being obtained with the transfer of higher numbers of cells. Suppressor cells are of great importance in the regulation of immune response and are either T cells (Gershon *et al.*, 1972; Morikawa *et al.*, 1977), B lymphocytes (Katz, Parker and Turk, 1974) or macrophages (Lichtenstein *et al.*, 1981). The suppression in our model was mediated by a lymphocyte enriched population and, within this, by the T lymphocytes obtained by nylon wool filtration. B lymphocyte enriched populations and glass-adherent cells did not suppress PFC. Depletion of macrophages did not abolish suppressor activity. That the suppressor cells in the JEV model are T lymphocytes has been confirmed in experiments where pretreatment of spleen cells with monoclonal anti-Thy 1.2 antibody and complement markedly reduced the suppressor activity. Such suppressor T cells have been demonstrated in dengue virus (Tandon *et al.*, 1979), influenza virus (Liew and Russell, 1980) and mouse-thymic virus (Cross, Morse and Asofsky, 1976) infections.

The present series of experiments suggest that the suppressor cells generated in JEV primed mice suppress PFC against DV equally effectively both *in vivo* and *in vitro*. Even in the Mackaness type of experiment the suppression was more or less similar for both the viruses. DV and JEV belong to the Flavi group of Toga viruses and have cross reacting group antigens. Recently we have shown that these suppressor cells do not suppress PFC against unrelated antigens viz SRBC and Coxsackie B_4 virus (Mathur *et al.*, unpublished). This shows that the suppressor cells are antigen-specific. We have found earlier an impairment of CMI against JEV and SRBC in JEV-infected mothers and their babies (Mathur *et al.*, 1983*b*), this may be due to the induction of suppressor cells. Antigen non-specific suppressor cells have been demonstrated both for humoral and cell-mediated immune response including DTH (reviewed by Kapp *et al.*, 1978), mixed lymphocyte reaction (Rich and Rich, 1974) as well as for inhibition of immune responses caused by mitogens (Pierce and Kapp, 1976). While antigen-specific suppressor cells have been shown in viral infection (Liew and Russell, 1980) and synthetic antigens (reviewed by Germain, 1980).

The suppressor activity was nearly absent at 1 and 2 weeks after JEV priming but appears sharply at 3 weeks and persists up to 6 weeks and then wears off. These cells mediate their regulatory function via liberation of a soluble suppressor

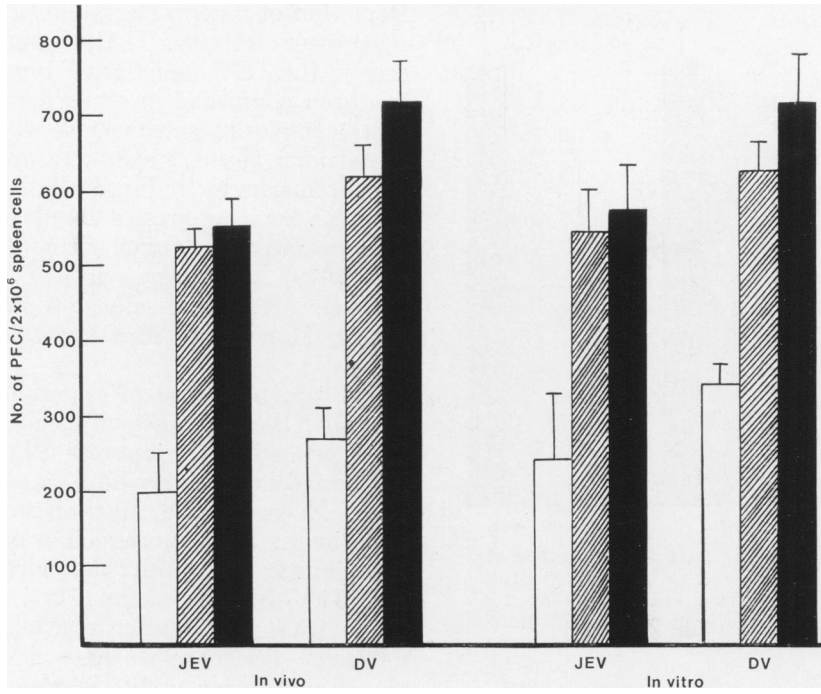

FIG. 7.—Virus group antigen specificity of the suppressor cell *in vitro* and *in vivo*. *In vitro*, spleen cell culture were stimulated with antigen at 0 h, followed 24 h later with inoculation of JEV primed spleen cells or normal spleen cells in control cultures. On days 3 and 4 IgM PFC against stimulating antigens were counted. *In vivo*, mice were given JEV and DV i.p. 10^3 LD_{50} followed 48 h later by JEVprimed spleen cells or i.v. normal spleen cells in controls. On days 5 and 6 IgM PFC were counted in spleen cells.

factor (Mathur *et al.*, unpublished). Earlier observations indicate that mice can be protected against JEV challenge by adoptive transfer of serum or spleen cells obtained at 1 and 2 weeks after JEV priming but not at latter periods (Mathur *et al.*, 1983*a*). Further, it was also noted that from third week onwards the primed mice had negligible CMI response as shown by leucocyte migration inhibition DTH and the IgM antibodies (Mathur *et al* 1983*a*,*b*). It is likely that the appearence of suppressor T cells at this period may be responsible for poor immune response from the third week onwards.

Suppressor cells can be generated by many factors, *viz.* by overloading of immune system with excess of antigen; by presentation of antigen by bypassing macrophages; or by the immune complexes. We have observed replication of JEV in the spleen and persistence of JEV in the thymic cells up to 17 weeks when the virus is inoculated i.p. in pregnant mice (Mathur *et al.*, 1982*a*). How far presence of this virus in the lymphoid tissue is responsible for the generation of suppressor cells is not known. We have observed presence of HAI as well as neutralizing antibodies in JEV primed mice from the first week onwards (Mathur *et al.*, 1983*a*). It appears likely that the antibodies may form complexes with the persisting virus antigen which may lead to induction of suppressor cells. The period of appearance of suppressor cells at the third week supports this conclusion. Further studies are in progress to investigate these aspects.

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