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Differential cyclophosphamide sensitivity of T lymphocytes of the dengue virus-induced suppressor pathway

M.I. Shukla and U.C. Chaturvedi

Virus Laboratory, Postgraduate Department of Pathology and Bacteriology, K.G. Medical College, Lucknow 226003, India

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Summary. Dengue type 2 virus (DV) induces a suppressor pathway in mice which involves sequential participation of three subpopulations of T lymphocytes viz. Ts_1, Ts_2 and T_3 cells. In the present study cyclophosphamide (CY) sensitivity of these cells have been investigated. The findings of the present study demonstrate that Ts_1 and Ts_2 cells are CY-insensitive while T_3 cells are CY-sensitive. This supports our earlier conclusion that T_3 may be the inducer cells of suppression.

Keywords: dengue virus, suppressor pathway, cyclophosphamide sensitivity, T lymphocytes

Cyclophosphamide (CY) has been extensively used in experimental animals to analyse the host response. It has been used to produce immunosuppression in experimental animals to convert a silent abortive infection into a lethal infection or to enhance its severity, accompanied by increased virus titres and an increased number of infected and destroyed cells in the target organs (Cole & Nathanson 1968; Robinson *et al.* 1969; Hurd & Heath 1975; Chaturvedi *et al.* 1977). A single injection of CY may suppress antibody formation (Chaturvedi *et al.* 1977, 1978) or aquisition of contact sensitivity (Turk 1964) or may enhance delayed-type hypersensitivity (DTH). These paradoxical responses depend on the period at which CY is given in relation to the antigen and the dose of the latter (Sy *et al.* 1977). CY has a short half-life and acts on immunologically competent and actively dividing cells (Santos 1967; Camiener & Wachter 1972). Two precursor subpopulations of T lymphocytes sensitive to CY are those of helper T cells for antibody production and the suppressor T cells for DTH (Gill & Liew 1978; Shand & Liew 1980). Enhanced DTH due to deletion of the precursors of suppressor T cells in CY-treated animals has been reported in contact sensitization to dinitrofluorobenzene (DNFB) (Sy *et al.* 1977); to sheep erythrocytes (Shand & Liew 1980) and in infections with influenza (Liew *et al.* 1979) and dengue (Pang *et al.* 1982) viruses.

In dengue type 2 virus (DV) infection of mice a three-step mechanism of antigen-specific suppression of antibody forming cells occur as follows: DV induces production of suppressor T cells (Ts_1) which produce a soluble suppressor factor (SF) which is transmitted by the macrophages to recruit another subpopulation of suppressor T cells (Ts_2) which produce a soluble prostaglandin-like product (SF_2) to recruit another sub-

Correspondence: U.C. Chaturvedi, Medical College Quarters, 50 Jagat Narain Road, Lucknow 226003, India.

population of T lymphocytes (T_3) to mediate suppression (Tandon *et al.* 1979; Chaturvedi & Shukla 1981; Chaturvedi *et al.* 1981, 1982; Shukla & Chaturvedi 1981*a*, 1981*b*, 1982, 1983, 1984; Shukla *et al.* 1982). In the present study an effort was made to investigate CY sensitivity of the three different subpopulations of T cells involved in the DV-induced suppressor pathway. It was observed that Ts_1 and Ts_2 cells are CY-insensitive while T_3 cells are CY-sensitive.

Methods and materials

Animals. Inbred Swiss albino mice aged 4 to 6 months have been used in the experiments.

Virus. Dengue type 2 virus (DV) strain P23085 used was in the form of infected adult mouse brain suspension as described elsewhere (Chaturvedi *et al.* 1977, 1978). A dose of 10^3 LD_{50} of the virus was used throughout the experiments.

Preparations of Ts_1 cells and the suppressor factor (SF). Ts_1 cells and the SF were prepared from DV-infected moribund mice spleens as described earlier (Chaturvedi *et al.* 1981; Chaturvedi & Shukla 1981; Shukla *et al.* 1982). In brief, mice were inoculated intracerebrally with 10^3 LD_{50} of DV. On the 10th day after inoculation when the mice were sick, spleens were harvested aseptically. A single cell suspension was prepared and used as Ts_1 suppressor cells. A 10% w/v homogenate of infected mice spleens was prepared in phosphate-buffered saline (PBS) pH 7. The homogenate was centrifuged at 2000 *g* to remove cell debris and then at 103 500 *g*. The clear supernatant was collected and was stored in small aliquots at −20°C. It was used as SF.

Preparation of Ts_2 cells and the SF_2. Preparation of Ts_2 cells has been described elsewhere (Shukla & Chaturvedi 1981*b*; Shukla *et al.* 1982). In brief a 1.0-ml cell suspension of normal mouse spleen (1×10^7 cells/ml) was treated with 0.4 ml of SF (1:100) for 1 h at 37°C. The cells were washed three times to remove unadsorbed SF and were cultured for 24 h. The cells and the culture fluid were separated and used as Ts_2 cells and SF_2 respectively. We have observed that SF-adsorbed macrophages can transmit the suppressor activity in DV-primed mice (Shukla & Chaturvedi 1983). To eliminate this the Ts_2-containing spleen cells were depleted of macrophages by treatment with carbonyl iron (Lymphocyte Separator Reagent, Technicon Instrument Corp., N.Y.) as described earlier (Tandon *et al.* 1979) and were used as Ts_2 cells after washing three times.

Cyclophosphamide treatment. Mice were treated with cyclophosphamide (Endoxan-ASTA, Khandelwal Lab. Pvt., Ltd) at doses of 200, 100 or 50 mg/kg of body weight. Cyclophosphamide (CY) was given intraperitoneally 2 days before inoculation of DV.

Preparation of spleen cell cultures. Mouse spleen cells obtained in single cell suspension were cultured in Eagle's minimum essential medium (MEM)-HEPES containing 10% fetal calf-serum, and 5×10^{-5}M 2-mercaptoethanol, penicillin and streptomycin. Cultures were prepared in 5-cm glass petri dishes with 4.0 ml cell suspension (5×10^6 cells/ml) and were incubated in an atmosphere of 5% CO_2 at 37°C (Shukla & Chaturvedi 1981*a*, *b*).

Assay of suppressor activity. The suppressor activity of various cell preparations and their products was screened *in vivo* or *in vitro*. For experiments *in vivo* the preparations were inoculated i.v. in mice given 10^3 LD_{50} DV i.p. 48 h earlier. For experiments *in vitro* normal mouse spleen cell cultures were inoculated with 10^3 LD_{50} DV at 0 h followed 24 h later with inoculation of the cell preparations or their products. DV-specific IgM-PFC were counted in spleen cells by the haemolysis-in-gel technique of Jerne & Nordin (1963) as described in detail elsewhere (Tandon & Chaturvedi 1977). The PFC were counted on days 6 and 7 of DV inoculation in *in vivo*

assay and on days 3 and 4 in *in vitro* assay. Mean values of 12–16 observations with standard deviations have been presented after deducting background PFC. The data were analysed using Student's *t*-test for *P* value.

Results

Effect of CY on production of Ts_1 cells.

The effect of cyclophosphamide treatment on the production of suppressor T cells (Ts_1) and SF production after stimulation with DV was screened *in vivo*. It was observed that suppressor cells, obtained from mice treated with the different doses of CY, suppressed 42 to 47% PFC which was similar to the effect produced by cells obtained from untreated mice (Table 1).

Effect of CY on production of SF

The suppressor activity of the SF prepared from mice treated with different doses of CY was compared with that prepared from untreated mice. The findings summarized in Table 2 show that the suppressor activity of the SF from drug-treated mice was 39 to 47% while that from untreated mice was 47 to 48% ($P > 0.01$).

Effect of CY on production of Ts_2 cells

Suppressor activity of Ts_2 cells prepared from spleen cells of mice pretreated with either of the three doses of CY was screened *in vivo*. As shown in Table 3, Ts_2 cells from mice pretreated with various doses of CY suppressed 42 to 46% PFC while cells from CY-untreated mice suppressed 45 to 46% PFC.

Effect of CY on production of SF_2

Spleen cells from mice treated with various doses of CY were induced to produce SF_2 and the suppressor activity of SF_2 thus prepared was screened *in vivo*. The findings presented in Table 4 indicate that SF_2 prepared from spleen cells of mice treated with CY suppressed 44 to 46% PFC and that prepared from spleen cells of CY-untreated mice suppressed 40 to 43% PFC.

Effect of CY on production of T_3 cells

The target of SF_2 is a subpopulation of T lymphocytes which we have termed T_3 cells (Shukla & Chaturvedi 1984). To observe the effect of CY on the production of T_3 cells, mice were inoculated with various doses of the drug i.p. followed 48 h later with DV i.p. and

Table 1. Effect of CY on induction of Ts_1 cells

Spleen cells of CY-treated mice (mg CY/Kg)*	PFC/2×10^6 spleen cells (after DV challenge)			
	Day 6		Day 7	
	Number	% suppression	Number	% suppression
200	480±37	42	477±34	44
100	460±33	44	466±36	45
50	453±26	45	449±29	47
CY-untreated	444±22	46	441±21	48
No cells	824±31	0	847±38	0

* Mice were treated with various doses of CY i.p. followed 48 h later with 10^3 LD_{50} of DV i.c. Spleen cells obtained from these mice on day 10 after DV inoculation were assayed for suppressor activity.

Table 2. Effect of CY on production of SF

SF from CY-treated mice (mg CY/Kg)*	PFC/2 × 10^6 spleen cells (after DV challenge)			
	Day 6		Day 7	
	Number	% suppression	Number	% suppression
200	485±25	41	496±40	40
100	472±38	42	503±36	39
50	436±23	47	460±33	45
CY-untreated	429±43	48	437±51	47
No SF	818±33	0	829±28	0

* Mice were treated with various doses of CY i.p. followed 48 h later with 10^3 LD_{50} DV i.c. SF was prepared from the spleen collected 10 days after DV injection and was assayed for suppressor activity.

Table 3. Effect of CY on induction of Ts_2 cells

Spleen cells of CY-treated mice (mg CY/Kg)*	PFC/2 × 10^6 spleen cells (after DV challenge)			
	Day 6		Day 7	
	Number	% suppression	Number	% suppression
200	468±36	44	466±43	45
100	477±34	43	461±31	46
50	484±40	42	461±45	46
CY-untreated	458±50	45	458±23	46
No cells	830±35	0	845±28	0

* Mice were treated with various doses of CY i.p. After 48 h spleen cells were obtained and induced with SF to produce Ts_2 cells as described in text and their suppressor activity was assayed.

Table 4. Effect of CY on production of SF_2

SF_2 from CY-treated mice (mg CY/Kg)*	PFC/2 × 10^6 spleen cells (after DV challenge)			
	Day 6		Day 7	
	Number	% suppression	Number	% suppression
200	443±20	46	452±29	46
100	461±28	44	448±24	46
50	451±30	44	461±29	44
CY-untreated	488±13	40	470±34	43
No SF_2	818±33	0	829±28	0

* Mice were treated with various doses of CY i.p. After 48 h, spleens were collected and the production of SF_2 was induced. The suppressor activity of SF_2 was assayed.

96 h later again with SF_2 i.v. It was observed that SF_2 suppressed 45% PFC in mice pretreated with 50 mg/kg of CY which was similar to that in mice not treated with CY (Table 5). SF_2 failed to produce suppression in mice which were treated with higher doses of CY.

These results were then confirmed by experiments *in vitro*. For these experiments mice were treated with various doses of CY i.p. After 48 h their spleens were collected and cell cultures prepared. The cultures were inoculated with DV followed 24 h later by inoculation of SF_2. As shown in Table 6, suppression of PFC by SF_2 was 6 to 10% in cell cultures prepared from mice given 100 or 200 mg/kg of CY, but 35 to 42% in cells from mice given 50 mg/kg of CY.

Table 5. Effect of CY on induction of T_3 cells: *in vivo* assay

Mice treated with CY (mg CY/Kg)*	SF_2 inoculation	PFC/2 × 10^6 spleen cells (after DV challenge)			
		Day 6		Day 7	
		Number	% suppression	Number	% suppression
200	+	769±39	6	783±27	6
100	+	700±45	15	700±21	16
50	+	453±28	45	456±28	45
—	+	457±30	41	470±34	43
—	−	818±30	0	829±28	0

* Mice were treated with various doses of CY i.p. After 48 h, mice were stimulated with 10^3 LD_{50} DV i.p. followed 48 h later with inoculation of SF_2. DV-specific IgM PFC were counted in the spleen on day 6 and 7 after DV inoculation.

Table 6. Effect of CY treatment on induction of T_3 cells: *in vitro* assay

Spleen cell cultures from CY-treated mice (mg CY/Kg)*	SF_2 inoculation	PFC/2 × 10^6 spleen cells (after DV challenge)			
		Day 3		Day 4	
		Number	% suppression	Number	% suppression
200	+	673±45	9	715±39	6
200	−	735±43	0	761±20	0
100	+	760±33	6	656±32	10
100	−	686±44	0	724±39	0
50	+	423±19	42	431±31	35
50	−	726±13	0	660±47	0
—	+	409±37	44	396±44	40
—	−	726±13	0	660±47	0

* Mice were treated with various doses of CY i.p. After 48 h spleen cell cultures were prepared from these mice and were stimulated with 10^3 LD_{50} DV followed 24 h later with inoculation of SF_2. DV-specific IgM PFC were counted on days 3 and 4.

Discussion

Synthetic antigen-induced suppressor cells and their products have been studied in depth and their suppressor pathways, involving up to three generations of T cells have been described (reviewed by Germain & Benacerraf 1981). The main parameters used in these studies to characterize the suppressor cells have been the surface phenotype, H-2 (I-J) and V_H restriction, antigen-specificity and CY-sensitivity of the cells. The only infectious agent used to delineate such a sequential suppressor pathway has been the dengue virus in which also three generations of the T cells have been established so far. DV-induced Ts_1 cells are Thy1.2$^+$, Ly23$^+$, species-restricted, antigen-specific (Tandon *et al.* 1979; Shukla & Chaturvedi 1981*b*; Chaturvedi *et al.* 1981, 1982; Shukla *et al.* 1982) and as shown in the present study are CY-insensitive. The Ts_2 cells are Thy1.2$^+$, Ly23$^+$, species-restricted, antigen-specific (Shukla & Chaturvedi 1981*b*, 1984; Shukla *et al.* 1982) and CY-insensitive (present study). The third generation of DV-induced T cells are Thy1.2$^+$, Ly1$^+$, antigen-specific and species-restricted (Shukla & Chaturvedi 1984) but is CY-sensitive as shown in the present study. One of the criteria suggested by Germain & Benacerraf (1981) in their unifying scheme of suppressor pathways is the sensitivity of the suppressor cells to CY. The findings of CY-insensitivity of DV-induced Ts_1 and Ts_2 cells do not disqualify them from being classed as a suppressor cell; Shand & Liew (1980), using sheep erythrocyte as antigen, have shown that the suppressor T cells for DTH and the helper T cells for the humoral response also are CY-sensitive. In DV model the indicator of suppression is IgM-PFC (humoral response); therefore, in the light of the findings of Shand and Liew (1980) and on the basis of Ly1$^+$ surface phenotype, the T_3 cell appears to be an inducer T cell.

All attempts to induce a DTH against DV in inbred Swiss albino mice were ineffective, and even pretreatment of such mice with CY did not elicit any DTH (unpublished data). Could this be due to simultaneous induction of CY-insensitive suppressor cells for DTH against DV? Simultaneous induction of suppressor cells for antibody production and DTH have been reported in a number of studies including those on sheep erythrocytes (Yamaguchi & Kishimoto 1978) and Japanese encephalitis virus (Mathur *et al.* 1983, 1984). The induction of DTH to DV in CY-treated mice, reported by Pang *et al.* (1982), may be due to strain difference of the mice used.

The third generation of T cells in keyhole limpet haemocyanin (KLH), dinitrophenyl (DNP) and azobenzenarsonate (ABA) induced suppressor pathways are the suppressor (Ts_3) cells (reviewed by Germain & Benacerraf 1981) while in the present model, the T_3 cell is an inducer cell having a surface phenotype Ly1$^+$. In various immune responses Ly1$^+$ T cells act as inducer or initiator cells and among them cells of the I-J$^+$ subset induce suppressor activity while cells of the I-J$^-$ subset induce B cell activity (Eardley *et al.* 1980). The suppression of PFC can be brought about in two ways; (i) either by direct suppression of B cells by the suppressor cells or their products, or (ii) by preventing the help through suppression of helper cells. With the data available in DV-model T_3 cells appear to induce suppression of B cell activity. Studies, including I-J phenotyping, are required to further characterize these cells and their mode of action.

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