Dengue Virus-induced Suppressor Factor Stimulates Production of Prostaglandin to Mediate Suppression

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SUMMARY

The immunosuppression in dengue type 2 virus (DV)-infected mice is mediated through secretion of a soluble suppressor factor (SF) produced by the T lymphocytes of the spleen. Activity of the suppressor cells was abrogated by pretreatment with indomethacin, indicating that suppression is mediated through prostaglandin (PG) or products of the PG synthetase pathway. The present study was undertaken to resolve the relationship between SF and PG. Treatment of mice with indomethacin did not affect production of SF. Therefore, PG is different from SF and the presence of PG is not essential for its production. Normal mouse spleen cells treated in vitro with SF produced PG which mediated suppression. Production of PG by these cells was abolished by pretreatment with anti-Thy 1.2 antibody and complement. It is concluded, therefore, that immunosuppression occurs in two phases: in the first step DV stimulates a subpopulation of T lymphocytes to produce SF, and in the second step SF induces a subpopulation of T lymphocytes to produce PG or products of the PG synthetase pathway which finally mediate suppression.

INTRODUCTION

Infection by a number of viruses is associated with immunosuppression in the host, but the mechanism of immunosuppression is not well understood (Woodruff & Woodruff, 1975). The immunosuppression observed in dengue type 2 virus (DV)-infected mice (Chaturvedi et al., 1977, 1978a) appears to be mediated by two factors, the cytotoxic factor (CF) and the suppressor factor (SF), both being produced in the spleen by T lymphocytes (Chaturvedi et al., 1980a; Tandon et al., 1979b). CF is a protein which destroys macrophages and a subpopulation of T lymphocytes, thus mediating immunosuppression non-specifically, and is effective against unrelated antigens like sheep red blood cells (SRBC) (Chaturvedi et al., 1980b, 1981b). The suppressor T lymphocytes induced in DV-primed mice (Tandon et al., 1979a) mediate suppression through a soluble substance, the SF (Chaturvedi & Shukla, 1981). We have purified and characterized the SF. It is a highly potent, heat-labile, trypsin-resistant, unstable at acid and alkaline pH, dialysable, low mol. wt. substance (Chaturvedi & Shukla, 1981). We have also observed that pretreatment of the suppressor cells with indomethacin or aspirin, the well known inhibitors of prostaglandin synthetase (Vane, 1976), abolishes the suppressor activity of the cells, thus showing that suppression may be mediated through prostaglandin (Chaturvedi et al., 1981a). We know of no previous similar study in virus infections. While further exploring the nature of SF a number of possibilities were taken into account, namely, SF is a prostaglandin (PG) produced by T cells on stimulation by DV; or SF is an intermediate product and stimulates the production of PG

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which ultimately results in immunosuppression; or DV stimulates the T lymphocytes to produce PG which induces a suppressor cell to produce the soluble SF. To examine these questions, experiments were done both in vitro and in vivo. Findings of the present study demonstrate that DV induces T cells to produce SF which in turn, induces production of PG which mediates immunosuppression.

METHODS

Animals. Inbred adult Swiss albino mice aged 4 to 6 months obtained from the colony of this department were used.

Virus. The details of the dengue type 2 virus (DV) strain 23085 and the experimental model have been described elsewhere (Chaturvedi et al., 1977, 1978a; Agrawal et al., 1978). Throughout this study a dose of 1000 LD$_{50}$ of the DV has been used. The other antigens used in specificity experiments were Japanese encephalitis virus (JEV) strain 78668A (Mathur et al., 1981) used as mouse brain suspension; Coxsackie B4 virus (Cox. B4) used as monkey kidney tissue culture fluid (Chaturvedi et al., 1978b); and SRBC kept in Alsever’s solution and washed three times before use (Chaturvedi et al., 1981b). The doses of the viruses were 1000 LD$_{50}$ (JEV) or 1000 TCID$_{50}$ (Cox. B4) and that of SRBC was 10$^8$ cells/culture.

Preparation of suppressor cells and suppressor factor. Suppressor cells develop in the spleen of mice infected intracerebrally with DV 8 to 11 days post-infection (Tandon et al., 1979a, b). Therefore, spleens were collected from the moribund mice at the above periods and a single cell suspension was prepared and used as suppressor cells. The details of the preparation of SF have been described previously (Chaturvedi & Shukla, 1981). Briefly, spleens collected from the virus-infected moribund mice on 8 to 11 days post-infection were homogenized in chilled phosphate-buffered saline (PBS) pH 7. The homogenate was centrifuged in the cold at 2000 g for 10 min and then at 103 500 g for 3 h. The supernate was collected, divided into small aliquots and stored at -20°C and used as suppressor factor (SF).

Preparation of spleen cells. The spleen cells were teased out in chilled minimum essential medium (MEM) with 10% foetal calf serum (Armour Pharmaceutical, Eastbourne, Sussex, U.K.). A single cell suspension was prepared and the viable nucleated cells were counted using trypan blue dye exclusion (Chaturvedi et al., 1978b).

Preparation of enriched spleen cell populations. The procedures of cell enrichment have been described previously (Tandon et al., 1979b; Chaturvedi et al., 1979, 1980a). Briefly, the macrophages were depleted by treating the spleen cell suspension with carbonyl iron (Lymphocyte Separator Reagent, Technicon Instruments, N.Y., U.S.A.) and a magnet. The T and B lymphocytes were separated by filtration of macrophage-depleted spleen cells through nylon-wool columns (Julius et al., 1973). Glass-adherent and non-glass-adherent cells were separated as described earlier (Chaturvedi et al., 1978b) by incubating whole spleen cell suspension in Petri dishes at 37°C in the presence of 5% CO$_2$.

Preparation of spleen cell cultures. Normal mouse spleen cells were cultured in 5 cm glass Petri dishes. Each Petri dish was seeded with 4 ml cell suspension (4 × 10$^6$ cells/ml) prepared in MEM–HEPES containing 10% foetal calf serum and 5 × 10$^{-5}$ M-2 mercaptoethanol. The cultures were incubated at 37°C in an atmosphere of 5% CO$_2$.

Drug treatment. Indomethacin (Sigma) was used to inhibit production of PG. The drug was dissolved in 0.85% NaCl containing 1% sodium bicarbonate (Lapp et al., 1980). Further dilutions of the drug were prepared in MEM. The cells were treated with 10$^{-6}$ M of the drug at 37°C for 1 h (Mattingly & Kemp, 1979) and washed three times. The drug treatment had no effect on the viability of the cells. The production of PG in mice was inhibited by administration of 0.3 mg of the drug intraperitoneally (i.p.) 24 and 2 h before the virus and then 0.1 mg i.p. at 48 h intervals.
Treatment with anti-thymocyte serum. To kill T lymphocytes the spleen cell suspension was treated with monoclonal anti Thy 1.2 antibody (New England Nuclear) and complement by the technique of Golub (1971) as described elsewhere (Tandon et al., 1979a). Serum, complement and cell controls were included with every test.

Detection of antibody-forming cells. The direct immunoglobulin M plaque-forming cells (PFC) against DV were counted by the localized haemolysis in gel technique of Jerne & Nordin (1963). The details of the technique have been described earlier (Chaturvedi et al., 1977; Tandon & Chaturvedi, 1977).

Experimental protocol. The experimental protocol was similar to that described earlier (Tandon et al., 1979a, b; Chaturvedi et al., 1981a; Chaturvedi & Shukla, 1981). Briefly, the suppressor activity of the preparation was studied using PFC against DV in the spleens of mice or in spleen cell cultures as indicator systems. For in vivo study, mice were given 1000 LD$_{50}$ DV i.p. followed 48 h later by intravenous (i.v.) injection of 0-25 ml SF (1:100 dilution). For in vitro study, normal mouse spleen cell cultures were prepared and inoculated with 1000 LD$_{50}$ DV at 0 h followed 24 h later by inoculation of 0-25 ml SF (1:100 dilution). The peak PFC response occurs on the sixth and seventh days after i.p. inoculation of mice with DV (Chaturvedi et al., 1977, 1981a; Tandon & Chaturvedi, 1977). In the spleen cell cultures the peak PFC response was observed on days 3 and 4 after DV inoculation (U. C. Chaturvedi et al., unpublished observations). In the present study, therefore, observations were recorded on these days. Each in vitro experiment was set up in triplicate and was repeated two or three times. For in vivo experiments, five to eight mice were used in each group of replicate experiments. From each mouse or culture multiple slides were prepared. The PFC count on the 2 days of the experiment (6 and 7 days in vivo and 3 and 4 days in vitro) were similar and therefore the values have been pooled for ease of presentation. Mean values with standard deviation have been presented after deducting background PFC. The data have been analysed by Student’s t-test for P-values.

RESULTS

Production of SF by T lymphocytes

To study the splenic cell type responsible for production of SF the cell populations were enriched and homogenized. The suppressor activity of the cell homogenate was screened in mice in vivo. The findings have been summarized in Table 1. The suppression produced by the homogenates of B lymphocytes was negligible, as was that produced by glass-adherent cells. On the other hand the suppression produced by the homogenates of T lymphocytes was 56%. The homogenates of normal mouse spleen cell preparations had negligible effect.

Suppression mediated by SF is antigen-specific

To study the antigenic specificity of the SF-mediated suppression spleen cell cultures were stimulated by different heterologous antigens and the effect of SF on their PFC counts examined. Table 2 presents the findings of this experiment. The SF suppressed 57% PFC against DV while those against JEV, Cox. B4 or SRBC were suppressed 12 to 16%. The suppression of PFC against the heterologous antigens was insignificant as compared to that observed for the PFC against DV.

Effect of treatment of suppressor cells with indomethacin

Spleens were collected from virus-infected moribund mice and a single cell suspension was prepared. The cells were divided into two aliquots; one was treated with indomethacin and the other with the diluent of the indomethacin. The cells were washed three times and their suppressor activity was assayed in vitro by inoculating $4 \times 10^6$ to $5 \times 10^6$ cells in the
Table 1. **Suppressor activity of homogenates of enriched spleen cell populations**

<table>
<thead>
<tr>
<th>Enriched spleen cell homogenate</th>
<th>Number (%)</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DV-infected mice cells†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrophage-depleted†</td>
<td>315 ± 38</td>
<td>54</td>
</tr>
<tr>
<td>Glass-adherent</td>
<td>625 ± 70</td>
<td>8</td>
</tr>
<tr>
<td>Non-glass-adherent</td>
<td>322 ± 29</td>
<td>53</td>
</tr>
<tr>
<td>T lymphocytes§</td>
<td>297 ± 56</td>
<td>56</td>
</tr>
<tr>
<td>B lymphocytes§</td>
<td>590 ± 45</td>
<td>13</td>
</tr>
<tr>
<td>Unfractionated</td>
<td>310 ± 52</td>
<td>55</td>
</tr>
<tr>
<td>No cells (control)</td>
<td>680 ± 25</td>
<td>0</td>
</tr>
<tr>
<td>Normal mice cells</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mice given DV i.p. followed 48 h later by homogenate of 10 × 10⁶ cells i.v. PFC against DV were counted on 6th and 7th days after DV inoculation. Mean value ± S.D. presented. Percent suppression was calculated from counts in control mice.
† Spleen cell homogenate prepared from moribund DV-infected mice.
‡ Macrophage depleted by carbonyl iron treatment and magnet.
§ T and B lymphocyte-enriched populations fractionated by nylon-wool column.

Table 2. **Antigenic specificity of the suppression mediated by SF**

<table>
<thead>
<tr>
<th>Stimulating antigen</th>
<th>SF</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>776 ± 33</td>
</tr>
<tr>
<td>Test</td>
<td>+</td>
<td>332 ± 34</td>
</tr>
<tr>
<td>JEV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>273 ± 29</td>
</tr>
<tr>
<td>Test</td>
<td>+</td>
<td>239 ± 30</td>
</tr>
<tr>
<td>Cox. B4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>376 ± 25</td>
</tr>
<tr>
<td>Test</td>
<td>+</td>
<td>332 ± 28</td>
</tr>
<tr>
<td>SRBC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>732 ± 36</td>
</tr>
<tr>
<td>Test</td>
<td>+</td>
<td>612 ± 30</td>
</tr>
</tbody>
</table>

* Spleen cell cultures were stimulated with the antigen at 0 h followed 24 h later with inoculation of SF in test cultures and diluent in control cultures. On days 3 and 4 cultures were harvested and IgM PFC against the stimulating antigen were counted. Percent suppression was calculated from that of controls of the antigen.

DV-stimulated cultures. Fig. 1 shows that DV-primed cells suppressed 40% PFC against DV. In preliminary experiments inoculation of normal mouse spleen cells to cultures had no effect on PFC counts. The suppression was abolished by pretreatment of the cells with indomethacin, thus showing that suppression is mediated through PG.

**Effect of indomethacin treatment of mice on production of SF**

We have observed that SF is similar to PG in many properties (Chaturvedi & Shukla, 1981). To determine whether SF and PG are identical substances we have examined the production of SF in mice whose capacity to produce PG was blocked by treatment with indomethacin. Mice treated with indomethacin i.p. and control mice given diluent in place of the drug, were inoculated i.c. with DV. When the mice were sick (days 8 to 11) the spleens were collected and their homogenates tested for suppressor activity in vivo. Findings presented in Table 3 show that the suppressor activity of the spleen homogenate of the
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Fig. 1. Abrogation of the suppressor activity of the suppressor cells in vitro by treatment with indomethacin. A, DV 10^3 LD_{50} inoculated into spleen cell cultures at 0 h followed 24 h later by inoculation of 4 × 10^6 to 5 × 10^6 suppressor cells treated with diluent; B, cultures inoculated with DV 10^3 LD_{50} followed 24 h later by inoculation of 4 × 10^6 to 5 × 10^6 suppressor cells treated with indomethacin; C, cultures inoculated with DV 10^3 LD_{50}. The cultures were harvested on days 3 and 4 and IgM PFC against DV were counted.

Table 3. Indomethacin treatment of mice has no effect on production of SF

<table>
<thead>
<tr>
<th>Spleen homogenate</th>
<th>PFC/2 × 10^6 spleen cells*</th>
<th>Suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug-treated† mice given DV i.c.</td>
<td>440 ± 24</td>
<td>46</td>
</tr>
<tr>
<td>Untreated mice given DV i.c.</td>
<td>430 ± 25</td>
<td>47</td>
</tr>
<tr>
<td>Normal mice</td>
<td>742 ± 40</td>
<td>9</td>
</tr>
<tr>
<td>None (control)</td>
<td>812 ± 18</td>
<td>0</td>
</tr>
</tbody>
</table>

* Mice were given DV i.p. followed 48 h later by spleen homogenate, 0.25 ml i.v. (1:10, w/v). PFC against DV were counted in the spleen on the 6th and 7th day after DV inoculation and mean value ± S.D. presented. Percent suppression was calculated from counts in control mice.

† Mice were treated with indomethacin, 0.3 mg i.p. 24 and 2 h before 10^3 LD_{50} DV i.c. and then 0.1 mg i.p. at 48 h interval. Spleens were collected from moribund mice at 8 to 11 days post-infection.

drug-treated mice was similar to that of untreated mice. This shows that SF is different from PG.

We then considered whether PG induces production of SF which in turn causes suppression. The direct approach would have been to inoculate pure PG to see if it induces production of SF. But pure preparations were not available and we had to draw conclusions indirectly from the above experiment. This showed that the presence of PG is not necessary for the production of SF.
Effect of SF on indomethacin-treated spleen cell cultures

In this experiment, performed in vitro, the production of PG was blocked by treatment of cultures with indomethacin. The aim was to show that if the suppression was mediated through PG the SF should have remained ineffective in indomethacin-treated cultures. The SF suppressed 57% IgM PFC against DV (P < 0.001) when added to DV-stimulated cultures (Fig. 2). The suppressor activity of the SF was abolished (5% suppression) if the culture cells were pretreated with indomethacin.

Effect of SF on indomethacin-treated mice

The above experiment was repeated in vivo by comparing the suppressor activity of SF in normal mice with that in indomethacin-treated mice. The findings of this experiment, summarized in Fig. 3, show that suppression of PFC by SF was 47% in untreated mice (P < 0.001) while in mice treated with indomethacin the suppression was 5% (P > 0.05).

These two experiments show that in vitro as well as in vivo the SF has no suppressor activity by itself, but the activity is mediated through prostaglandin.

SF stimulates normal mouse spleen cells to produce prostaglandin

The following experiments were done to determine whether SF needs the presence of PG to mediate suppression or whether it also stimulates production of PG by normal mouse spleen cells. The mouse spleen cells, with and without prior exposure to indomethacin, were stimulated with SF in vitro for 1 h and then the cells were cultured for 24 h at 37 °C after washing three times to remove unadsorbed SF. The culture supernates collected after 24 h were screened for suppressor activity in vivo in mice. Data presented in Table 4 show that
SF-induced PG mediates immunosuppression in dengue

Table 4. SF induces normal mouse spleen cells to produce PG

<table>
<thead>
<tr>
<th>Culture supernatant*</th>
<th>Number (PFC/2 × 10⁴ spleen cells)</th>
<th>Suppression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen cells stimulated by SF‡</td>
<td>421 ± 20</td>
<td>44</td>
</tr>
<tr>
<td>Drug-treated spleen cells stimulated by SF§</td>
<td>697 ± 40</td>
<td>7</td>
</tr>
<tr>
<td>ATS-treated spleen cells stimulated by SF‖</td>
<td>760 ± 38</td>
<td>-1</td>
</tr>
<tr>
<td>ATS-treated spleen cells treated with drug and then stimulated by SF</td>
<td>766 ± 38</td>
<td>-1</td>
</tr>
<tr>
<td>Drug-treated spleen cells</td>
<td>744 ± 29</td>
<td>1</td>
</tr>
<tr>
<td>Untreated spleen cells (control)</td>
<td>752 ± 29</td>
<td>0</td>
</tr>
</tbody>
</table>

* Spleen cells were cultured at 37 °C for 24 h and then cell-free culture supernatant was collected.
† Mice were given DV i.p. followed 48 h later by 0.25 ml of culture supernatant i.v. PFC against DV were counted on the 6th and 7th days after DV inoculation. Percent suppression was calculated from the counts in control mice.
‡ 1 × 10⁸ spleen cells were treated with 0.4 ml (1:10 dilution) of SF at 4 °C for 1 h. The cells were washed three times and then cultured.
§ Drug-treated spleen cells stimulated by SF.
‖ Spleen cells pretreated with 10⁻⁶ M-indomethacin were treated with SF.

Supernatants of spleen cell cultures stimulated by SF suppress PFC in vivo by 44% as compared to that from untreated cultures. In contrast, the suppression produced by culture supernatants of the indomethacin-treated cells stimulated by SF was 7% (Table 4). These experiments provided further evidence that SF stimulates normal spleen cells to produce prostaglandin to mediate suppression.

SF stimulates T lymphocytes to produce prostaglandin

Our earlier studies have shown that the suppression is mediated through T cells (Tandon et al., 1979 b; Chaturvedi et al., 1981 a). Therefore, an attempt was made to determine whether SF stimulates T lymphocytes to produce prostaglandin. The normal mouse spleen cells were treated with monoclonal anti-Thy 1.2 antibody and complement which killed 40% of spleen cells. The cells were washed and the above experiment was repeated. It was observed that the suppression was abolished by treatment with anti-Thy 1.2 antibody (Table 4) indicating that SF stimulates T lymphocytes to produce PG.

DISCUSSION

The present study was undertaken to resolve the relationship between the SF and the PG in DV-infected mice. To answer different questions we have made use of the drug indomethacin, a well known irreversible inhibitor of the enzyme prostaglandin synthetase (Vane, 1976) which has been widely used in vitro and in vivo to inhibit production of PG (Webb & Nowowiejski, 1977; Mattingly & Kemp, 1979; Lapp et al., 1980; Fulton & Levy, 1980). Indomethacin has no other known effect on lymphocyte metabolism (Webb & Nowowiejski, 1977). We have observed that the SF is produced even when production of PG is inhibited. Therefore, PG is neither needed for production of SF nor is it similar to SF. The data have shown that SF stimulates normal spleen cells to produce PG and further that PG is produced by T lymphocytes (i.e. anti-Thy 1.2 antibody + complement-sensitive cells). In DV-infected mice the suppressor cells are T lymphocytes (Tandon et al., 1979 a, b) which supports our findings described here of the production of SF by T cells.
The present study thus demonstrates that suppression in DV-infected mice is brought about in two steps. In the first step, DV stimulates T cells to produce the soluble suppressor factor (SF). The second step involves induction of T lymphocytes by SF to produce PG or products of the PG synthetase pathway which finally mediate suppression. Measurement of PG levels both in vitro and in vivo is required to confirm induction of its production by SF. PG of E type has been shown to inhibit effectively the immune response and production of lymphokines, thus mediating a feedback regulatory loop involving two types of cells (for review, see Waksman, 1979). A perusal of the literature reveals that among leukocytes, monocytes and macrophages are the main producers of PG on stimulation by various agents (Gemsa et al., 1978, 1980; Friedman et al., 1979), whereas there is only one report of involvement of lymphocytes (Webb & Osheroff, 1976). The data presented here show that T lymphocytes are also capable of producing PG on stimulation by SF. Inhibition of PG does not affect SF production and pure SF induces production of PG. Therefore, two subpopulations of T lymphocytes appear to be involved, one producing SF and the other producing PG. Further clarification of this question will need direct assay of different T lymphocyte subpopulations to determine which produces SF and which PG. The cooperation between these subpopulations is mediated through signals provided by a soluble substance, SF; therefore, direct cell-to-cell contact is not needed.

We have observed that the immunosuppression mediated by SF–PG is DV antigen-specific as shown by suppression of PFC against DV but not those against Japanese encephalitis or Coxsackie B4 viruses or sheep erythrocytes. How PG mediates suppression and why sometimes this is antigen-specific is not fully known. The suppressor activity of PG has been attributed to intracellular increase of cyclic AMP (cAMP). Novogrodsky et al. (1979) have suggested that PG mediates selective suppression of lymphocyte responses via increased intracellular cAMP. They have further suggested that selectivity depends upon: (i) nature of the mitogen; (ii) difference in molecular properties of cAMP-dependent protein kinases of the responding cell; and (iii) difference in the stage of differentiation or activation of the immune system. On the other hand, Mattingly et al. (1979) have shown that the release of PG is controlled by a mechanism different from the production of PG. Furthermore, Mattingly & Kemp (1979) have suggested that some antigen specificity is involved with PG-induced suppression as the signal for release comes from specific antigen committed cells. It has also been observed that the suppressor factor, produced in mice treated with GAT or GT, is associated with a small quantity of the antigen or their fragments. This suppressor factor induces a second generation of T lymphocytes (TS2) which are absolutely specific to the antigen which stimulated initial suppressor cells (for review, see Tada & Okumura, 1979). In our model the signal for production of PG comes from specific DV antigen-primed cells, which probably results in antigen specificity in PG-mediated immunosuppression. SF appears to stimulate a de novo synthesis of PG, since indomethacin completely blocked its production. The mechanism of SF-stimulated PG production by T lymphocytes remains to be investigated.

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**References**


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