Inhibition of Prostaglandin E$_2$ Restores Defective Lymphocyte Proliferation and Cell-Mediated Lympholysis in Recipients After Allogeneic Marrow Grafting

By Hans-G. Klingemann, Mang-So Tsoi, and Rainer Storb

Prostaglandins are said to influence T and B cell function by inhibiting the generation of interleukin 2 (IL 2) and the formation of suppressor lymphocytes. After bone marrow transplantation, patients usually have a profound immunodeficiency that persists in recipients with chronic graft-vs-host disease (GVHD) and generally resolves in long-term survivors without GVHD. In vitro tests of lymphocyte function such as allogeneic mixed lymphocyte culture (MLC) and cell-mediated lympholysis (CML) have been shown to be impaired in many patients. We postulated that prostaglandin E$_2$ (PGE$_2$) plays a role in the impaired in vitro tests. To test this hypothesis, we studied in vitro tests in the presence of PGE$_2$, antagonists, indomethacin, and anti-PGE$_2$ antisera with cells from 22 short-term patients (<100 days postgrafting) and 32 long-term survivors with or without GVHD. Results show that blockade of PGE$_2$ release by indomethacin and anti-PGE$_2$ significantly (<0.01) enhanced the MLC (+67%) and the CML responses (10.5%) of cells from long-term survivors with chronic GVHD but not from those of long-term, stable recipients. No enhancement of MLC and CML activity was observed with cells from donors of long-term recipients. In patients shortly after marrow grafting, enhancement in the MLC was not significant. However, CML activity in this patient group was significantly increased (+12.5% in recipients with no GVHD, 8.5% in those with acute GVHD, P < .01). Indomethacin also suppressed the activity of nonspecific suppressor cells in patients with chronic GVHD. When cells from patients with chronic GVHD were treated with recombinant IL 2 and IL 2 combined with indomethacin, it was possible to get an additional augmentation of lymphocyte proliferation after the addition of indomethacin to IL 2-treated cultures. Thus it is very likely that PGE$_2$ inhibits T lymphocyte proliferation, not exclusively by inhibition of IL2 production or activity. We conclude that PGE$_2$, among other factors, may play a role in the pathogenesis of the immunodeficiency after transplantation. PGE$_2$ does not act primarily by interfering with IL 2 but presumably by inducing a suppressor-like activity.

EXPERIMENTS in a number of species have demonstrated that some immune functions are inhibited by prostaglandin E$_2$ (PGE$_2$) such as lymphocyte proliferation, activation of cytotoxic lymphocytes, cell-mediated lympholysis (CML), and B cell proliferation. In murine and human models, monocytes produce sufficient amounts of PGE$_2$ to mediate this inhibitory effect. There are several explanations for the way in which PGE$_2$ exerts its inhibitory activity. Evidence is growing that PGE$_2$ is bound to a functional subset of human T cells that leads to their activation as suppressor cells. Other data suggest that PGE$_2$ interferes with interleukin 2 (IL 2) production or activity and/or that it reduces Ia expression on monocytes.

Many patients after bone marrow transplantation (BMT) have a profound immunodeficiency related to impaired function of both B and T cells. This immunologic defect may be severe and life threatening, is seen in all patients within the first few months of transplantation, and persists in long-term survivors who have chronic graft-vs-host disease (GVHD). In marrow recipients, reduced lymphocyte proliferation can be restored by exogenous IL 2. The same is true for the diminished CML in patients with acute GVHD, but not for those with chronic GVHD.

It was the aim of this investigation to evaluate whether PGE$_2$ contributes to the impaired mixed lymphocyte culture (MLC) reactivity and CML of cells from patients after BMT. The results obtained show that in vitro blocking of PGE$_2$ action by two distinct inhibitors improves lymphocyte proliferation and CML activity in cells from marrow graft recipients. In addition, cells from patients with chronic GVHD were treated with IL 2 or indomethacin, or both. The augmenting effect on allogeneic lymphocyte proliferation by each substance was additive. This observation makes it very likely that PGE$_2$ inhibits T lymphocyte proliferation not only by inhibition of IL 2 production or activity. Our results point to an indomethacin-sensitive suppressor cell that inhibits lymphocyte proliferation and whose effects cannot be overcome by exogenous IL 2 alone.

MATERIAL AND METHODS

Patients and donors: Marrow transplantation. Cells from 61 allogeneic marrow recipients (8 with aplastic anemia, 38 with acute myelogenous leukemia, 11 with acute lymphoblastic leukemia, and 4 with non-Hodgkin’s lymphoma) were studied 22 to 948 days after allogeneic marrow transplantation. The patients’ ages ranged from 2 to 49 years; 34 were male and 27 female. Details of the transplantation procedures have been described previously. Patients with aplastic anemia were conditioned for grafting with cyclophosphamide, 50 mg/kg, on each of four successive days. Those with malignancies were conditioned for grafting with a combination of cyclophosphamide, 60 mg/kg, on each of two successive days and total-body irradiation. Evidence for marrow engraftment was obtained by monitoring the peripheral blood count, marrow cellularity, and cytogenetic markers. Patients were divided into four groups based on the time of testing and the presence or absence of GVHD (first figure gives the number of patients tested in MLC; the figure in parentheses refers to the number of patients tested for CML): 11 (10) short-term patients with acute GVHD tested between 22 and 94 days after BMT, 16 (11) short-term patients without GVHD tested...
PGE\(_2\) INHIBITION IN PATIENTS AFTER BMT

between 33 and 100 days after BMT, 22 (11) long-term patients with chronic GVHD tested between 294 and 858 days, and 12 (7) long-term patients without chronic GVHD tested between 312 and 948 days after BMT. In all long-term recipients, cells from marrow donors were tested simultaneously with those of their respective recipients to serve as normal syngeneic controls. The protocol for this study was subject to initial approval and subsequent annual reviews by the Human Subjects Review Committee of the Fred Hutchinson Cancer Research Center and the University of Washington. All participants in the study signed committee-approved consent forms.

Reagents. Indomethacin (Sigma Chemical Co, St Louis, cata-

cal No. 1-7378) was dissolved in 95% ethanol at 4 mg/mL and
diluted to 20 g/mL in RPMI 1640 medium (GIBCO, Grand

Island, NY) before use. It was determined that addition of ethanol at concentrations equal to those present in the dissolved drug had no detectable effect on our cultures. Indomethacin was used in concen-

trations of 0.01 \(\mu\)g/mL (3 \(\times\) 10\(^{-8}\) mol/L) to 10 \(\mu\)g/mL (3 \(\times\) 10\(^{-7}\) mol/L). Since 1 \(\mu\)g/mL gave optimal enhancement under our experimental conditions as determined by a dose-response curve, we chose this as the final concentration for all experiments added on day 0 to the cultures. Rabbit anti-PGE\(_2\) was purchased from Sigma (No. P 6765) and dissolved in phosphate-buffered saline at 5 \(\text{mg}/\text{mL}\). Lyophilized PGE\(_2\) (5Z, 11X, 13E, 15S)-11,15-dihydroxy-9,11-e-

prosta-5,13-dien-1-oic acid purchased from Sigma (No. P 5640) was

dissolved at 95% ethanol at a concentration of 0.1 \(\mu\)g/mL

(2.8 \(\times\) 10\(^{-8}\) mol/L) and was used at a final concentra-
tion of 10\(^{-8}\) mol/L, in RPMI 1640 medium. The final concentra-
tion of ethanol in the cultures was <0.1%.

Recombinant IL 2 (lectin free) was purchased from Amgen

Biologics (Thousand Oaks, Calif, No. 01010) and dissolved in 10

\(\mu\)mol/L Tris-buffered saline, pH 8. Twenty units were added to each

culture.

Purification of lymphocytes and culture media. Peripheral

blood mononuclear cells (PBMC) were obtained from heparinized

whole blood by density gradient centrifugation using lymphocyte

separation medium (Bio-technics) as described.\(^{24}\) The mononuclear

cells were suspended in RPMI 1640 supplemented with 2 mmol/

L-glutamine (GIBCO), 25 mmol/L HEPES buffer (GIBCO), 1%

penicillin-streptomycin solution (GIBCO), and 20% heat-inacti-

vated pooled human male serum. All cultures were incubated at

37 °C in humidified 5% CO\(_2\) in an air atmosphere.

Mixed lymphocyte cultures. Unidirectional allogeneic MLC

were performed in quadruplicate of U-bottomed plates (Costar,

Cambridge, Mass) with 1 \(\times\) 10\(^6\) responder and an equal number of

irradiated (16 Gy) stimulator cells in 0.2 mL RPMI 1640 culture

medium. After incubation for six days, cultures were pulsed with 2

\(\mu\)Ci of \(^{3}H\)-thymidine (New England Nuclear, Boston) and

incubated for six hours. The cultures were harvested and processed with an automated microplate harvester (Cambridge Technology, Cam-

bridge, Mass), and incorporation of radioactivity was measured in a

liquid scintillation counter and expressed as the mean counts per

minute per quadruplicate cultures \(+ SD\). The variation among

microcultures did not exceed 15%. To exclude any direct effect on

the responding or stimulator cells, controls were run with responding

and stimulator cells using media alone or alternatively with indom-

ethacin, anti-PGE\(_2\), or IL 2. Response to allogeneic stimulator

cells after addition of indomethacin, anti-PGE\(_2\), or IL 2 was

expressed as the net counts per minute of cells with the additive

minus counts per minute of cells without the additive.

CML assay. To use as few cells as possible from most patients,

the indirect CML assay was carried out by a micromethod according
to Zarling et al\(^{25}\) with slight modifications. MLC were set up as described with 2 \(\times\) 10\(^6\) responder and stimulator cells per well.

Target PBMC (5 \(\times\) 10\(^6\)/2 mL) from the same donor as the stimula-
tor cells were prepared at the same time as the stimulator and were

incubated under the same conditions as the MLC. On day 6 of MLC, the target cells were concentrated in 0.1 mL of heat-inactivated fetal
calf serum and were labeled with 300 \(\mu\)Ci of \(^{51}Cr\) (Na\(_{2}\)CrO\(_4\), New

England Nuclear) for 60 minutes at 37 °C. After incubation and

washing, they were resuspended at 1 \(\times\) 10\(^7\)/mL in RPMI 1640 with

5% fetal calf serum. An aliquot (0.1 mL) of the supernatant was

removed from each MLC culture, and an equal volume of the labeled

target cells (1 \(\times\) 10\(^5\)) was added to each culture, making the ratio of effector to target cells 10:1. After centrifugation (140 g for

five minutes) the plates were incubated for six hours at 37 °C in a

humidified 5% CO\(_2\) incubator.

An aliquot of supernatant was then harvested from each culture

and its radioactivity determined in a gamma counter. The percent-
ge of \(^{51}Cr\) release was calculated from the formula: (experimental

release – autologous release) \times 100/(maximum release – auto-

logous release).

Maximum release values were obtained by adding 0.15 mL of

0.1% Nonidet P40 to 0.1 mL of target cells. Controls with autologous

stimulator cells were performed with medium, indomethacin, or

anti-PGE\(_2\), which were added at the initiation of the assay. In each

experiment three different dilutions (5:1, 10:1, 50:1) of effector cells

were tested to determine whether a log linear relationship existed

between the ratio of effector cells to target cells and the amount of

\(^{51}Cr\) release. Since we never observed decreased results at different

effector-target ratios (E:T) ratios that did not appear at the 10:1 E:T ratio,

only the results obtained with this ratio are presented.

Non-specific suppressor cell assay. PBMC of the recipient

(chimera cells of donor marrow origin) were cocultured with lymphocytes obtained from the marrow donor and irradiated third-party

stimulator cells in a mixed lymphocyte reaction. Control cultures

consisted of chimera cells alone responding to third-party stimu-

lators and donor cells alone responding to the same stimulators. The

percent suppression in any single test was calculated according to the

formula: 1 – [(DuP + DuD + PuP)/2] \times 100 – percent suppression

(D, donor PBMC; P, patient PBMC; U, unrelated irradiated lympho-

cytes). A positive percentage indicated the degree of suppression

of donor response by patient cells. A percentage greater than 25% was

used as a guideline for detecting cases with suppression.\(^{23}\)

Statistical methods. The Wilcoxon two-sided signed rank test

was applied to paired responses of patient PBMC before and after in

vitro treatment. Values for allogeneic induced lymphocyte prolifera-
tion from patients were considered significantly different from

normal if an individual patient value fell <2 SD below the mean for

the control group. Comparisons between groups were done with

Student's t-test.

RESULTS

Improvement of allogeneic MLC of cells from marrow

recipients by exogenous indomethacin. Allogeneic lympho-

cyte proliferation was tested in the MLC with cells from

four groups of marrow recipients. Reactivity of cells from

patients was compared with that of cells from their respective

donors. The results are presented in Table 1 and Fig 1. Cells from

short-term patients and patients with chronic GVHD showed a markedly reduced \(^{3}H\)-thymidine uptake as compared with long-term patients without GVHD or healthy donors. Exogenous indomethacin increased the response rate in cells of patients with chronic GVHD (P < .01) and in

long-term stable recipients (P < .05). There was no significant

beneficial effect in the cells of patients shortly after transplantation, regardless of whether GVHD was present. There was also no correlation between immunosuppressive medication after marrow grafting (ie, cyclosporine, metho-
trexate, antithymocyte globulin) and impaired MLC responses.

Since indomethacin is not a specific PG synthetase inhibitor, we compared its effect with that obtained with an anti-PGE₂ antiserum using cells from eight patients with chronic GVHD. Results in Fig 2 illustrate that ³H-thymidine incorporation in cells from these patients was enhanced by anti-PGE₂ in a manner similar to that seen with indomethacin.

Restoration of CML activity with indomethacin in recipients shortly after grafting and in patients with chronic GVHD. CML activity was diminished with cells from all patient groups regardless of the time after transplantation. Results are summarized in Table 2 and Fig 3. Before exogenous indomethacin was added, CML activity was lowest in recipients with acute and chronic GVHD. The addition of indomethacin improved CML by 8.6% and 10.6%, respectively, but normal activity could be achieved only in patients with chronic GVHD and in short-term patients with no GVHD, but not in recipients with acute GVHD. CML activity was also enhanced by indomethacin in long-term patients with no GVHD (+6.3%) and in healthy controls (+4.4%). This improvement was statistically not significant (Wilcoxon signed rank test). As in the MLC, the antiserum against PGE₂ was equally effective in enhancing CML activity compared with indomethacin (Fig 2).

Effect of the addition of PGE₂. PGE₂ (10⁻³ mol/L) suppressed proliferation of cells from healthy individuals in the MLC as well as their activity in the CML (range of suppression, 35% to 68% in both tests, results of eight controls). When indomethacin (1 μg/mL) was added to the cultures together with PGE₂, PGE₂-induced suppression was not reversed (results not shown).

Additive effects of IL 2 and indomethacin on the allogeneic MLC of patients with chronic GVHD. PBMC of nine patients with chronic GVHD were stimulated with irradiated allogeneic cells in the presence and absence of IL 2, indomethacin, and IL 2 plus indomethacin (Table 3). The addition of IL 2 to PBMC cultures from patients augmented the proliferation by a mean of 60% (range, 7% to 131%). The addition of indomethacin to the same cultures augmented the MLC by a mean of 68% (range, 27% to 156%). However, adding the combination of IL 2 and indomethacin increased proliferation by a mean of 172% (range, 21% to 534%). This increase was statistically significant (P < .01) if compared with the increases observed with each single agent.
Table 3. Augmentation of Allogeneic MLC of Cells From Patients With Chronic GVHD Receiving an Optimal Stimulating Dose of IL 2, Indomethacin, or IL 2 + Indomethacin. The Percent Increase is Given as Compared With the MLC Without Exogenous IL 2 or Indomethacin.

<table>
<thead>
<tr>
<th>UPN</th>
<th>Underlying Disease</th>
<th>Test Day Postgrafting</th>
<th>Current Medication</th>
<th>Percent Increase of CPM</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+ Il 2</td>
</tr>
<tr>
<td>705</td>
<td>AML</td>
<td>2,865</td>
<td>-</td>
<td>40</td>
</tr>
<tr>
<td>1,598</td>
<td>ALL</td>
<td>1,176</td>
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<td>21</td>
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<tr>
<td>2,337</td>
<td>ALL</td>
<td>416</td>
<td>-</td>
<td>131</td>
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<td>420</td>
<td>P</td>
<td>117</td>
</tr>
<tr>
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<tr>
<td>2,416</td>
<td>CML</td>
<td>616</td>
<td>P</td>
<td>7</td>
</tr>
<tr>
<td>2,515</td>
<td>CML</td>
<td>261</td>
<td>-</td>
<td>98</td>
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<tr>
<td>Mean ± SE</td>
<td></td>
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<td>60 ± 15</td>
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</tbody>
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Abbreviations: A, azathioprine; P, prednisone; UPN, unique patient number; Indo, indomethacin; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia.

The percent increase of cpm was calculated as follows: (average cpm of indomethacin-treated cells/average cpm of control cultures) - 1.0 × 100.

**DISCUSSION**

To understand the underlying causes of immunodeficiency commonly seen in patients early after allogeneic BMT and in long-term survivors with chronic GVHD, we had earlier analyzed various cellular immune functions during different stages of T cell activation of marrow recipients and found that (1) their monocytes usually had normal function26,27 and could produce IL 128; (2) their T cells were immunologically defective18,20,28 and were deficient in IL 2 production29; and

**Generation of nonspecific suppressor cells is decreased after indomethacin.** We tested the PBMC of patients with chronic GVHD for nonspecific suppressor cells and found in four recipients nonspecific suppressor activity greater than 25%. Exogenous indomethacin decreased suppression in all of these cases significantly (P < .001). In three of these patients suppressor activity was reduced to ≤25% (Fig 4).
T-cell proliferation is mediated at two levels. Cell Immunol 61:52, 1980

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GVHD.22 In addition, nonspecific suppressor cells were found in some recipients, with chronic GVHD24 thought to contribute to the depressed T cell immunity and subsequently to a higher incidence of late infections.29 These findings and the reports from others showing that human IL 2 production was regulated by PGE2,3 and that PGE2 might be involved in suppressor T cell induction,9,11,12,30,31 prompted us to investigate the role of PGE2 in the immunodeficiency after marrow grafting.

We made use of the findings of several laboratories that PGE2 was able to inhibit human lymphocyte proliferation1-4 and that blocking of PGE2 effects by indomethacin or antiserum against PGE2 could restore the in vitro immune response.32-34 We were able to demonstrate that the impaired MLC and CML activity with cells from many recipients after marrow transplantation, especially in long-term patients with chronic GVHD, was significantly improved by exogenous indomethacin and also by an anti-PGE2 antiserum. Our demonstration that a specific anti-PGE2 serum could mimic the effect of indomethacin suggests that PGE2, rather than other prostaglandins produced by adherent cells, is the main immunosuppressive factor. These findings suggest a role for PGE2 in the severe immunodeficiency seen in patients after marrow grafting.

What are the mechanisms of PGE2-mediated immunosuppression? Some authors believe that PGE2 directly suppresses human lymphocyte proliferation by interfering with both the production of IL 2 and IL 2-dependent proliferation.15,14 Others8,11,32 claim that PGE2 suppressed lymphocyte responses indirectly by activation of a suppressor cell. Our present data showing that the activity of nonspecific suppressor cells in patients with chronic GVHD can be significantly inhibited by indomethacin points to PGE2 as a possible inducer of the suppressor activity. Furthermore, Fisher et al10 recently demonstrated PGE2 receptors on human suppressor lymphocytes.

As to the cause of increased PGE2 effects, two explanations are possible: an enhanced sensitivity of grafted lymphocytes to normal PGE2 levels or an increased production of PGE2. As shown by Lapp et al35 PGE2 synthesis by mouse macrophages is induced during GVHD reaction. But they also observed that the response to indomethacin was abrogated about 15 to 20 days after GVHD induction. The fact that the augmentation of the allogeneic MLC with indomethacin did not reach significance in our patients with acute GVHD might be explained by this observation, since most of our patients had their disease for more than 2 weeks before being tested.

In addition to our observation with indomethacin only, it was possible to enhance the allogeneic MLC by combining indomethacin and IL 2. The response was superior to the effect of either agent alone. A recent paper by Chouaib et al36 confirmed this observation. Exogenous IL 2 restored PGE2-induced inhibition of T cell proliferation only partially. They showed that PGE2 downregulates the expression of the transferrin receptor, which has been correlated with cell proliferation in normal lymphocytes.

PGE2 is known to increase the intracellular concentration of adenosine 3'5'-cyclic monophosphate (cAMP).37 By testing other drugs that enhance intracellular cAMP levels and reduce transferrin receptor expression, Chouaib et al36 concluded that cAMP might be the mediator of the inhibitory effect of PGE2 on lymphocyte proliferation. Our observation of a reduction in vitro activity of nonspecific suppressor cells by indomethacin and the fact that it was possible to get an additional augmentation of lymphocyte proliferation after the addition of indomethacin to IL 2-treated cultures suggests the existence of an indomethacin-sensitive suppressor cell whose effect can apparently not be overcome by IL 2 alone. A similar additive effect of indomethacin and IL 2 was observed in cancer patients whose PBMC were stimulated by phytohemagglutinin.38,39

Recent observations in the mouse model showed no increase in the incidence of GVHD by administration of IL 2 in vivo while simultaneously improving the defective immunologic responsiveness of recipient mice.39 Based on our in vitro studies in man, it might be possible that a combination of IL 2 and indomethacin is superior compared with IL 2 alone in correcting a defective immune response after experimental marrow grafting without aggravating GVHD.

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Inhibition of prostaglandin E2 restores defective lymphocyte proliferation and cell-mediated lympholysis in recipients after allogeneic marrow grafting

HG Klingemann, MS Tsoi and R Storb