Generation of CFU-C/Suppressor T Cells In Vitro: An Experimental Model for Immune-Mediated Marrow Failure

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T cells were derived from the bone marrow of 8 healthy donors and fractionated, according to their receptors for the Fc fragment of IgG, into T0 cells and Tc cells. These were then cocultured with autologous or allogeneic bone marrow cells in agar in the CFU-C assay. No significant suppression of colony formation could be detected. Total T, T0, and Tc cells were then incubated for 18 hr with PWM, washed, and cocultured with bone marrow cells. PWM-treated Tc cells showed no significant CFU-C suppressor activity, whereas PWM-treated total T and T0 cells inhibited colony formation of both autologous and allogeneic marrow cells. The supernatant of PWM-treated total T and Tc cells also inhibited colony formation. PWM alone enhanced colony formation. The results of this study indicate that normal T cells can be activated in vitro to become CFU-C suppressor cells after PWM stimulation, and that this effect is mediated by T cells with the Fc receptor for IgG.

SOME PATIENTS with severe aplastic anemia (SAA) are thought to have an immune-mediated hemopoietic failure, based on the fact that removal of T lymphocytes from the bone marrow enhances colony formation (CFU-C, BFU-E)4,5 and that some of them achieve an autologous hematologic reconstitution after immunosuppressive treatment.6,7 We have shown in a previous study that T cells with IgG receptors (Tc cells) are responsible for CFU-C suppression detected in autologous and allogeneic cocultures in patients with SAA in remission.8 We also showed in the same study that normal Tc cells are not capable of suppressing colony formation of autologous or allogeneic marrow cells.9 We then asked the question: Is it possible to activate normal Tc cells in vitro and switch on the CFU-C suppressor activity? Tc cells have been shown to suppress B-cell differentiation in vitro only after antigen stimulation (pokeweed mitogen, PWM).8 We therefore elected to activate T cells derived from the marrow of healthy donors with PWM, and then test the ability of unfractionated, Tc cells, and Tc cells to inhibit colony formation.

MATERIALS AND METHODS

Bone marrow donors. Eight healthy untransfused volunteers served as donors. Bone marrow blood was aspirated from the posterior crests in heparinized syringes.

Bone marrow cells. These were run first on Ficoll-Hypaque gradient (800 g), and the light density cells (LD) recovered and washed 3 times in McCoy's medium. The cells were then incubated in plastic Petri dishes at 37°C for 30 min in 5% CO2 with McCoy's culture medium supplemented with 20% fetal calf serum (FCS) to remove adherent cells. Nonadherent cells were washed out with the supernatant.

Rosetting techniques. LD bone marrow cells, depleted of adherent cells, were then rosetted with sheep erythrocytes treated with neuraminidase.8 E-rosetting cells were separated from non-E-rosetting cells on two sequential Ficoll-Hypaque gradients as previously described.6 E-rosetting cells were then incubated with ox erythrocytes coated with rabbit anti-ox erythrocytes IgG, and then run on a density gradient.8 Two subpopulations were thus obtained: nonrosetting cells (Tc cells) and rosetting cells (Tc cells). The latter were freed of ox erythrocytes by lysing with ammonium chloride buffer and then run on a density gradient.9

Light microscope and cytochemical analysis. Tc and Tc cells were centrifuged onto glass slides using a cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, Pa.). The slides were then stained with May-Grünwald-Giemsa for morphological studies, after having been fixed in methanol for 15 min. For the evaluation of a-naphthyl-acid esterase (ANAE), the cells were fixed and incubated for 60 min at 37°C with hexazotized pararosaniline and a-naphthyl acetate at pH 5.8.10 Acid phosphatase activity was determined on fixed cells according to the technique of Goldberg and Barks.11

Immunofluorescence staining. Viable cell suspensions of T-cell subsets were reacted with goat antibodies to human immunoglobulins, conjugated with fluorescein isothiocyanate (F/P ratio 1.5) (Kallestad Laboratories Inc., Chaska, Minn.), washed, and examined in suspension with a Zeiss microscope equipped with incidence illumination at 480 nm.

Pretreatment of T cells with pokeweed mitogen (PWM). This was performed by incubating total T, Tc, and Tc cells (2 x 106 cells in 0.2 ml of culture medium with 2 μg of PWM) for 18 hr at 37°C in 5% CO2.

In vitro bone marrow cultures. These were then set up to test the suppressor activity of total T, Tc, and Tc cells. Target cells consisted of 105 autologous or allogeneic bone marrow cells deprived of T cells (T-depleted bone marrow cells). These target cells were plated according to the technique of Pike and Robinson12 together with 105 unfractionated T cells and T-cell subsets, both untreated or preincubated with PWM. Tc cells were also plated in the feeder layer in one set of experiments at the same concentration (105 cells). Controls consisted of spontaneous colony formation of normal marrow cells deprived of T lymphocytes, and of unfractionated T cells or T-cell subsets. Suppression was calculated as observed/expected growth x 100, and expressed as percentage of expected.
growth. Target cells (normal marrow cells) were also cocultured with irradiated (1500 rad) PWM-treated T₀⁻ cells (10⁵ cells), and with the supernatants of PWM-treated T₀⁻, and T₀ cells (undiluted 0.1 ml, and at different dilutions 1:10, 1:100). Finally, PWM alone (2 g in 0.1 ml) was added to normal marrow cells to exclude a possible inhibitory activity of the mitogen on colony growth. Cultures were read on day 10 and scored for colonies (over 50 cells) and clusters (less than 50 cells).

RESULTS

Markers to Establish the T-Cell Nature of T₀⁻ and T₀ Cell Fractions

Isolated T-cell subsets were again rosetted with sheep erythrocytes treated with neuraminidase: over 95% of the cells formed E-rosettes. Immunofluorescence staining with antiimmunoglobulin antisera proved that less than 1% of the cells had detectable surface immunoglobulins.

In addition, morphological and cytochemical investigations were performed to exclude that macrophages contaminating the E-rosetting cell populations might have been selected with the T₀⁻ fraction through binding of IgG-coated ox erythrocytes via an Fe receptor. Morphologically, the T-cell fractions contained over 98% of cells with the characteristics of previously described T₀⁻ and T₀ cells, respectively. T₀⁻ cells had an abundant cytoplasm with small azurophilic granules and a low nuclear/cytoplasmic ratio. T₀⁻ cells were composed of small cells with a large nucleus and a high nuclear/cytoplasmic ratio. The cytoplasm of these cells contained no granules. Cytoplasmic markers for monocytes-macrophages, such as acid phosphatase and strong homogeneous monocyte-type ANAE positivity, were not detected in purified T-cell subsets.

Colony Formation of Normal Marrow Cells

This was 45 ± 18 colonies/10⁵ cells and 52 ± 14 after T-cell depletion. T cells, T₀⁻, and T₀⁻ fractions (10⁵ cells) produced no colonies.

Total T, T₀⁻, and T₀⁻ Cells

Total T, T₀⁻, and T₀⁻ cells showed no significant inhibitory activity when cocultured with autologous or allogeneic (T-deprived) marrow cells. (Figs. 1 and 2). T₀⁻ cells inhibited colony formation by 10% ± 4% in autologous combinations, and by 15% ± 5% in allogeneic combinations. Total T cells had no inhibitory activity. T₀⁻ cells, on the contrary, exhibited a moderate enhancing activity in autologous combinations (7% ± 2.6%) and no effect in allogeneic cocultures (Figs. 1 and 2).

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**Fig. 1.** Suppression of T-cell subsets on autologous (T-deprived) marrow cells. T₀⁻ and T₀ cells show no suppression on colony formation prior to pokeweed mitogen (PWM) treatment (90% ± 4% and 107% ± 26% of expected growth, respectively). After PWM treatment, T₀⁻ cells show a clear CFU-C suppression (34% ± 22%), whereas T₀ cells show no suppression and in some cases an enhancement of colony growth (94% ± 33%). Columns represent means ± SD of 8 experiments. The difference between untreated and PWM-treated T₀⁻ cells (PWM/T₀⁻) is statistically significant (p < 0.001).
Pokeweed mitogen (PWM) Treated $T_G^+$ Cells

These showed marked CFU-C/suppression on both autologous (66% ± 22%) and allogeneic (62% ± 26%) marrow cells. The difference between untreated and PWM-treated $T_G^+$ cell suppression was statistically significant ($p < 0.001$) (Figs. 1 and 2). Unfractionated T cells exhibited a moderate suppressor activity after PWM stimulation (41% ± 8%). $T_G^-$ cells pretreated with PWM failed to suppress colony formation, and in some instances, enhanced colony formation in both autologous and allogeneic combinations (Figs. 1 and 2). There was no significant difference between untreated and PWM-treated $T_G^-$ cells in their ability to suppress CFU-C ($p > 0.1$).

The different CFU-C/suppression of PWM-treated $T_G^+$ and $T_G^-$ cells was statistically significant at the $p < 0.001$ level, for both autologous or allogeneic combinations. $T_G^+$ cells pretreated with PWM were capable of suppressing colony formation when incubated with the target marrow cells in the upper agar layer, but also if plated in the underlayer (62% and 58% suppression, respectively).

Irradiation

Irradiation with 1500 rad of $T_G^+$ cells pretreated with PWM abrogated their suppressor activity. Indeed, inhibition of colony formation was less than 5% after irradiation.

Supernatant

The supernatant of PWM-treated $T_G^+$ cells showed a clear suppressor effect (Fig. 3), whereas this was not so when the supernatant of PWM-treated $T_G^-$ cells was added to the marrow cell cultures (Fig. 3). The supernatant of PWM-treated total T cells exhibited an inhibitory activity on colony growth (32% ± 21%) but to a lesser extent than $T_G^+$ cells. The difference between the CFU-C/suppressor effect of the supernatants from $T_G^+$ (64% ± 35%) and $T_G^-$ cells (20% ± 28%) was statistically significant at the $p < 0.05$ level. One should point out that PWM alone enhanced colony formation (24% ± 9%) and that PWM was not removed from the supernatants of PWM-treated cells, therefore, the inhibitory activity of supernatants, particularly if derived from $T_G^+$ cells, would have been probably increased in the absence of PWM.

The suppression of colony formation was dependent on the concentration of supernatants, derived from PWM-treated $T_G^+$ cells, used in the cultures: 64% ± 35% at a dilution of 1:1, 30% ± 12% at 1:10, 2% ± 8% at 1:100.
Marrow failure in patients with severe aplastic anemia (SAA) may be due to a true stem cell defect, to a defective microenvironment, or to an immune-mediated suppression of hemopoiesis. The third mechanism has been recently supported by the demonstration of suppressor cells in the peripheral blood and in the bone marrow of some patients with SAA. Macrophages have been reported to have CFU-C/suppressor activity in patients with SAA, and claims for a B-lymphocyte-induced marrow failure have also been made. In a number of patients, the CFU-C/suppressor activity would appear to be mediated by T cells. Coculture studies involving T-cell subsets have also been carried out in SAA patients and would indicate that only T cells with IgG receptors (T<sub>G</sub><sup>+</sup> cells) possess CFU-C/suppressor activity. On the contrary, T<sub>G</sub><sup>-</sup> lymphocytes derived from normal donors do not suppress colony formation in vitro.

However, T<sub>G</sub><sup>-</sup> cells do not circulate in the activated form in vivo, except in particular conditions, such as in the fetus, and must be triggered in vitro in order to express their inhibitory activity on pokeweed mitogen (PWM) induced plasma cell formation.

Mitogens such as PHA and Con-A have been recently shown capable of inducing suppressor T cells against BFU-E and CFU-E in both animals and man, and alloantigen stimulation during mixed lymphocyte reactions in mice would appear to activate suppressor cells after day 3 of culture.

In the present report we have shown that PWM stimulation is capable of producing CFU-C/suppressor T cells, and this activity is mediated by T<sub>G</sub><sup>-</sup> cells. Indeed, unstimulated T cells failed to suppress colony formation in 8 healthy donors, whereas total T and T<sub>G</sub><sup>-</sup> cells suppressed colony formation after interaction with PWM. On the contrary, cells lacking Fc receptors for IgG (T<sub>G</sub><sup>-</sup> cells) failed to suppress colony growth after PWM stimulation.

Three conclusions can be drawn from these experiments. In the first place, nonspecific activation of normal T<sub>G</sub><sup>-</sup> cells leads to the generation of suppressor T<sub>G</sub><sup>-</sup> cells reactive against both autologous and allogeneic marrow cells. It is unlikely that CFU-C/suppression is mediated by non-T<sub>G</sub><sup>-</sup> cells, since contamination of the T<sub>G</sub><sup>-</sup> cell fraction with other cells such as monocytes proved to be less than 1%. Secondly, T<sub>G</sub><sup>-</sup> cells behave differently from T<sub>G</sub><sup>-</sup> cells, since they do not suppress, and in some cases enhance, colony formation after treatment with PWM. In the third place, normal T<sub>G</sub><sup>-</sup> lymphocytes are not activated to suppress hematopoiesis in vivo, or alternatively, their inhibitory activity is so low to be hardly detected in the CFU-C assay, unlike some patients with SAA in
which T\(_{G}^{+}\) cells are activated in vivo and suppress efficiently colony formation.\(^3\)

One is also tempted to speculate that the switch from a resting T\(_{G}^{+}\) cell to an activated CFU-C/suppressor TG cell may be triggered in vivo after antigen stimulation, as shown in vitro. However, the chance of such an activation to occur in vivo is quite high, particularly during viral and bacterial infections or after vaccinations, when compared to the chance of developing an immune-mediated aplastic anemia. One would therefore have to postulate the existence of a particular proneness of single individuals of developing suppressor T cells, and thus, immune-mediated marrow failure. If this were the case, we would have been unsuccessful in activating T\(_{G}^{+}\) cells in all of our 8 healthy donors, although the degree of suppression expressed by PWM-treated T\(_{G}^{+}\) cells varied from 40% to 100%. Another possible explanation would be that activation of suppressor T\(_{G}^{+}\) lymphocytes, though occurring frequently, is under control in healthy individuals. Indeed T\(_{G}^{+}\) cells suppressing hematopoiesis may be present in small numbers at any given time. During infections, activated T\(_{G}^{+}\) cells would be greatly increased in number but short lived, and their effect on hematopoiesis, of short duration, as suggested by the transient pancytopenias or single line defects reported to follow viral infections in animals\(^{29-34}\) and in man.\(^{35-42}\) On the contrary, suppressor T\(_{G}^{+}\) cells would be self-maintained in patients who develop aplastic anemia, either because unhooked from control, or because of the lack of control.\(^1\) The result is the persistence of suppressor T cells and the development of acute or progressive hemopoietic failure, with severe injury to the stem cell pool. CFU-C/suppressor T cells may be detected for periods of time exceeding 2 yr also in patients who recover their marrow function.\(^3\)

The fact that both autologous and allogeneic CFU-C can be inhibited by these cells would indicate that the abnormality of SAA/T\(_{G}^{+}\) lymphocytes lies in their uncontrolled in vivo activation and not in the loss of tolerance for self antigens, nor in the hyperreactivity to altered stem cell antigens. It is also unlikely that cells secreting colony-stimulating factor (CSF) may be the target for suppressor T cells, since patients with SAA are known to have high levels of CSF\(^{43}\) and also of burst promoting activity (BPA).\(^{44}\)

Two sets of experiments indicate that CFU-C/suppression is mediated by a soluble factor: first the supernatant of T\(_{G}^{+}\) cells, incubated overnight with PWM, suppresses efficiently colony formation, whereas PWM alone enhances colony growth. Second, T\(_{G}^{+}\) cells are capable of inhibiting CFU-C when cocultured with the target marrow cells in the upper agar layer, but also when plated in the feeder layer, suggesting the production of a soluble agent. It is, however, unclear whether the in vitro activation of T\(_{G}^{+}\) cells triggers the production of different suppressor factors, specific for different targets or systems (such as the PWM-driven B-lymphocyte differentiation or the granulocyte-macrophage colony formation), or alternatively whether the suppressor agent or agents are non-specific.

CFU-C/suppression mediated by T\(_{G}^{+}\) cells can be abrogated with irradiation, as already shown in T\(_{G}^{+}\) cells derived from SAA/patients.\(^3\)

In conclusion, bone marrow T\(_{G}^{+}\) lymphocytes from normal donors can be induced in vitro to suppress colony (CFU-C) formation after interaction with PWM and thus to behave like T\(_{G}^{+}\) cells derived from some patients with severe aplastic anemia. Although the physiologic significance of the in vitro activation of T\(_{G}^{+}\) cells remains to be assessed, further studies on the role of T\(_{G}^{+}\) cells, and possibly also of other T-cell subsets, in the regulation of hematopoiesis may prove rewarding.

REFERENCES


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