Cells With Fcγ Receptors From Normal Donors Suppress Granulocytic Macrophage Colony Formation

By G. Spitzer and D. S. Verma With the Technical Assistance of S. Abramowitz and B. Tomasic

We investigated the role of normal human marrow cells with Fc receptors for IgG (Fcγ+) on autologous granulocyte-macrophage colony (GM-CFC) formation. It was found that Fcγ+ normal human marrow cells, both with (E+) or without receptors for sheep erythrocytes suppressed GM-CFC at as low a concentration as 0.25 × 10⁶ cells/ml of culture. A similar effect was observed with E+Fcγ+ but not E−Fcγ+ peripheral blood cells. Suppression by Fcγ+ cells did not require mitogen activation and was not inactivated by irradiation (2000 R). This report presents a new in vitro regulatory mechanism for GM-CFC growth in normal donors.

MATERIALS AND METHODS

Specimen Collection

Bone marrow was obtained from normal human volunteers. The marrow cells used were obtained by several posterior iliac crest punctures under local anesthesia. Aspirates were limited to 1 ml in volume to reduce the risk of peripheral blood T-cell or other mononuclear cell contamination. Peripheral blood in some instances was obtained from the same normal volunteers. Before acquiring a specimen for experimentation, we obtained a signed informed consent as required by the Surveillance Committee on Human Experimentation at The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute at Houston. The specimens were collected in polystyrene tubes (Corning Glass Works, Corning, N.Y.), containing 0.3 ml preservative-free heparin (1000 U/ml) in 1.7 ml phosphate-buffered saline (PBS).

Cell Separation Procedure

An outline of the cell separation sequence is given in Fig. 1. Briefly, bone marrow or peripheral blood was first fractionated by Ficoll-Hypaque gradient centrifugation to obtain light density (LD) cells (density 1.077 g/ml).

Preparation of Adherent and Phagocytic-Cell-Depleted Populations

LD cells were depleted of adherent and phagocytic cells by incubating them in 75 cm² plastic flasks (Corning Glass Works, Corning, N.Y. no. 2551-10) at a concentration of 4 × 10⁶ cells/ml. The day prior to experimentation, flasks had been pretreated with 5 ml of fetal calf serum (FCS) overnight. The FCS was removed prior to addition of cells. The cells were suspended in single strength, alpha-modified minimal essential medium (α-MEM, K C Biological, Kansas City, Kans.). Cells were subjected to the adherence procedure for 1 hr. During the last half hour of the adherence procedure, 2 ml carbonyl iron was added. Nonadherent cells were then removed and subjected to Ficoll-Hypaque density gradient centrifugation (density 1.077 g/ml) to remove phagocytic cells. This LD, nonadherent (NA), nonphagocytic (NØ) fraction was then subjected to E+ cell removal.

Removal of T Cells

To separate E-rosetting cells, we used a modification of the method described by Weiner et al. Briefly, 1 ml of a 5% sheep erythrocyte suspension was incubated for 1 hr at 37°C in a neur-
Fig. 1. The sequence of marrow fractionation. Marrow or blood was fractionated by Ficoll-Hypaque density gradient centrifugation to produce light density cells (LD), and then to produce a LD nonadherent nonphagocytic population (LDNANO). T cells were separated by E-rosette formation, leaving a T-cell-depleted (LDNANOT) target. From the pellet, T cells were further fractionated into Fcy’ and Fcy fractions. Non-T Fcy’ populations were obtained from LDNANOT cell populations.

Culture Procedure

The culture procedure has been described previously. Briefly, the marrow preparations were cultured in equal volumes of double strength α-MEM and 30% FCS and 0.6% agar (Bacto-agar, Difco, Detroit, Mich.), giving a final concentration of 0.3% agar with single strength α-MEM and 15% FCS. All cultures were plated in triplicate and incubated for 8-14 days in a fully humidified atmosphere of 7% CO2 and air at 37°C.

Culture Scoring

Cultures were scored on day 8 with use of an Olympus dissecting microscope at ×25-40. They were analyzed for total number of colonies (aggregates of 40 or more cells) per plate. The final incidence was the mean colony incidence from each plate.

Statistics

Differences between the results of experiments were examined by a two-sample t test. All comparisons were two-sided.

RESULTS

GM-CFC Incidence in Marrow Fractions After Removal of T’, Fcy’, and T’ Fcy’ Cells

These investigations were initiated to determine if the marrow T-cell populations regulated GM-CFC differently than did the blood T cells. Marrow pooled
from 1-ml aspirates was fractionated according to the protocol in Fig. 1 and cultured for GM-CFC. The GM-CFC incidence in some of these fractions from the initial experiments is shown in Figs. 2 and 3. In several instances, there were large increments in GM-CFC growth with removal of T cells (experiments 1, 2, 4, and 5). In experiments 1 and 4, the absolute number of GM-CFC recovered after T-cell removal was greater than that measured in the NAN# fraction (225% and 315%, respectively). Initial experiments also included removal of T+ Fcγ+ cells (Fig. 1). In all instances where Fcγ+ cells were 20% of the T-negative population, there were relative increases in the GM-CFC recovery and in experiments 2 and 3 absolute increases in GM-CFC recovery.

Suppression of GM-CFC Growth by T Fcγ+ and LDNAN# T Fcγ+ Marrow Cells

Readdition of the subpopulations removed suggested that these results were not artifacts secondary to
<table>
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<th>Expt.</th>
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Fig. 4. Bone marrow T-cell modulation of GM-CFC growth. E-rosette-positive (T⁺), E-rosette-positive cells with (T⁺ Fcγ⁺) or without (T⁺ Fcγ⁻) Fcγ receptors were added in proportions ranging from 0.25-1 × 10⁶ to 0.5 LDNAN∅ T⁺ target cells. In this particular example, removal of whole T cells was associated with a significant increment in GM-CFC growth (expt. 2, Fig. 3). Both T⁺ and T⁺ Fcγ⁻ showed suppression of GM-CFC. The p values for the individual experiments are given. Results are expressed as mean ± SE of triplicate determinations.

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Fig. 5. Bone marrow T-cell modulation of GM-CFC growth. E-rosette-positive cells (T⁺) with (T⁺ Fcγ⁺) or without (T⁺ Fcγ⁻) Fcγ receptors were added in proportions ranging from 0.5-2 × 10⁶ to 1 × 10⁶ LDNAN∅ T⁺ target cells. In this particular example, removal of whole T cells was not associated with any increment in GM-CFC growth (expt. 6, Fig. 3). Only T⁺ Fcγ⁻ showed suppression of GM-CFC not T⁺ or T⁺ Fcγ⁺. Results are expressed as mean ± SE of triplicate determinations.
or T⁺Fcγ⁺ cells enhanced GM-CFC (p value <0.05 for 0.5 × 10⁵ T⁺ and T⁺Fcγ⁺). However, addition of T⁺Fcγ⁺ suppressed GM-CFC growth (p <0.02 with 1 × 10⁵ and p < 0.01 for 2 × 10⁵ T⁺Fcγ⁺ cells).

Figure 6 shows data suggesting that non-E-rosetting cells with Fcγ⁺ markers from adherent phagocytic T-cell-depleted marrow populations (LDNANOT T Fcγ⁺) also suppress GM-CFC growth. Suppression of GM-CFC was uniform (all p values <0.05), occurred at cell numbers of only 0.25 × 10⁵ per culture, and was not sensitive to irradiation (2000 R). Removal of Fcγ⁺ cells from T-cell-depleted population in experiments 4 and 5 of Fig. 3 was not associated with significant increases in GM-CFC growth. However, the percentage of Fcγ⁺ cells in these fractions in these experiments was only 10% and 15% compared with 25%–35% in other experiments.

Differences in Suppression of Marrow and Blood T-Cell Populations

Having shown that marrow Fcγ⁺ cells, both E-rosetting and non-E-rosetting, could suppress GM-CFC growth, we next tried to determine whether there was a difference in suppressive activity between peripheral blood and marrow E-rosetting populations. Figures 7 and 8 detail one such experiment typical of two others. Peripheral blood T cells, regardless of Fcγ⁺ status, increased GM-CFC growth above that stimulated with HPCM alone (Fig. 7). However, LDNANØ T⁺Fcγ⁺ populations from marrow of the same volunteer suppressed growth, and LDNANØ T⁺Fcγ⁺ fractions enhanced growth more than that of blood (Fig. 8).

Comparison of Suppression of GM-CFC by E-Rosette-Positive and Negative Marrow Fcγ⁺ Cell Populations

We next performed further experiments to determine which was the most active marrow suppressor, T⁺Fcγ⁺ or T⁺Fcγ⁻ cells. T⁺Fcγ⁺ cells at higher numbers per culture (10⁵) suppressed GM-CFC growth the most (Fig. 9).

Figure 10 shows that removal of T⁻Fcγ⁺ cells from the target population did not remove the ability of either T⁺Fcγ⁺ or T⁻Fcγ⁺ cells to suppress GM-CFC growth. Other experiments (data not given) showed that T cells within the target populations (NANØ Fcγ⁻ target) did not prevent the suppression of either type of Fcγ⁺ population.

Comparison of Suppression of GM-CFC by E-Rosette-Negative Marrow and Blood Fcγ⁺ Cell Populations

Last, we looked for suppression by LDNANOT T⁻Fcγ⁺ Fcγ⁺ cells from blood. Table 1 shows seven such experiments examining this. Peripheral blood T⁻Fcγ⁺ cells regularly suppressed GM-CFC from marrow LDNANOT T⁻Fcγ⁺ fractions when added directly to
### FCγ CELLS—NORMAL MARROW AND BLOOD—GM-CFC INHIBITION

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<th>GM-CFC</th>
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Fig. 7. Peripheral blood T-cell modulation of GM-CFC growth. Autologous peripheral blood T cells or T⁺ FCγ⁻ and T⁻ FCγ⁺ cells were added in cell proportions ranging from 0.25-1.0 x 10⁶ to 0.25 x 10⁶ LDNANOT bone marrow cells. No suppression was seen; instead, in some instances GM-CFC growth was significantly enhanced. The p values for individual experiments are given. Results are mean ± SEM of triplicate cultures.

Agar cultures. This was invariable at 0.25 x 10⁶ cells/ml. In five of these seven experiments marrow and blood cell populations were compared, and in experiments 1, 3, 4, and 5 peripheral blood T⁺ FCγ⁺ were significantly more suppressive than marrow. Not shown from all these experiments is that peripheral blood T⁻, T⁻ FCγ⁺, T⁺ FCγ⁻, and T⁻ FCγ⁻ were again not suppressive, and T⁻ FCγ⁻ populations were particularly stimulatory to GM-CFC growth.

### Cellular Composition of Marrow FCγ⁺ Cell Population

Morphological examination of the T⁺ FCγ⁺ cells revealed that 95% or more of the cells were peroxidase-
Fig. 9. The comparison of Fcγ' E-rosette-positive and negative cell population suppression of LDNANOT targets. T⁺ Fcγ' and LDNANOT Fcγ' cells were added in proportions of 0.25, 0.5, and 1.0 x 10⁶ cells to 0.25 x 10⁵ LDNANOT targets. Both Fcγ' populations produced approximately 50% inhibition. The p values for individual experiments are given. Results are expressed as the mean ± SEM of triplicate cultures.

Fig. 10. The comparison of Fcγ' E-rosette-positive and negative cell population suppression of LDNANOT Fcγ' targets. The same populations as in Fig. 8 were added to 0.25 x 10⁶ LDNANOT Fcγ' cells. Again, T⁺ Fcγ' were more than equal in suppressive ability to T⁻ Fcγ'. p Values for individual experiments are given. Results are the means ± SEM of triplicate cultures.
negative and that they were predominately lymphoid. However, the amount of cytoplasm was greater and the nucleus more irregular than lymphocytes contained within the T' Fcγ population. The marrow T' Fcγ population was variable between experiments. The percentage of peroxidase-positive cells ranged between 30% and 70% (mean 45%). A large proportion of the cells were lymphoid (mean 40%). No correlation could be made between the suppression induced by the T' Fcγ' and the proportion of lymphoid or peroxidase-positive cells.

**DISCUSSION**

This article presents the first demonstration that Fcγ' cells from normal marrow, whether E' or E, can suppress GM-CFC growth without prior mitogen stimulation. Bacigalupo stated that T' Fcγ' cells derived from marrow of aplastic anemia patients, but not normal marrow, suppressed GM-CFC growth. However, no data were reported on adding these cells to Fcγ'-cell-depleted normal marrow. Furthermore, they used peripheral blood leukocytes as a stimulus for GM-CFC growth and achieved only an average of 52 colonies/10^6 cells from T-cell- and monocyte-depleted marrow. It is possible that the extra colonies activated by HPCM are those that are sensitive to suppression without PWM activation.

Ascensao et al. similarly reported that unactivated T' or T' Fcγ' peripheral blood cells did not suppress GM-CFC growth of T-cell- and monocyte-depleted autologous marrow targets. However, with T' Fcγ' fractions, they produced 30% inhibition, a value that apparently did not reach statistical significance. Furthermore, a mixture of T' Fcγ' and T' Fcγ' caused significant suppression. PWM stimulation of whole T cells or T-cell subsets also caused significant suppression. These authors did not study marrow T-cell subpopulations, except to state that T cells with IgM receptors were low compared to those in peripheral blood.

It is possible that the separation procedure activated these Fcγ' cells. However, in a number of instances, removal of T' Fcγ' caused absolute increments in GM-CFC recovery, suggesting that these cells, before in vitro contact with immunoglobulin, were active in suppression. Furthermore, as stated above, other workers have not noticed suppression of GM-CFC in normals by T Fcγ' cells when peripheral blood T cells are separated by identical procedures. These arguments still do not totally exclude this possibility. This could only be eliminated by future studies that would positively select for this cell by other surface markers or negatively select for it from marrow T cells by Fcμ rosette formation or other T-cell markers. These experiments are not possible until we delineate the surface markers of the one or several types of cells involved in this GM-CFC inhibition.

This report is the only documentation of suppression of GM-CFC growth by a T' Fcγ' cell from normal marrow unexposed to mitogen. This suppression can reach significance at relatively low numbers of Fcγ' cells per culture (0.25 × 10^6). Ascensao produced 90% or greater inhibition with either PWM plus T cells or their subpopulations, or alternately, the conditioned media from these subsets. The identity or dissimilarity of the inhibitors produced by nonactivated or mitogen-stimulated T-lymphocyte populations must to be established.

A major question is the real nature of the T' Fcγ' suppressive cell in our studies. In view of the fact that T' Fcγ' cells were equally suppressive of GM-CFC, the results may be secondary to contamination of our marrow T' Fcγ' fraction by this cell. We are uncertain at this time of the nature of the cell within the T' Fcγ' population that is responsible for suppression. This fraction contains promyelocytes, myelocytes, and lymphoid forms. However, the T' Fcγ' fraction did contain greater than 90% T cells (E rosette), no nonspecific esterase-positive cells, and only 1%-5% peroxidase-positive cells. If suppression was secondary to contamination by a non-E-rosette-positive cell, then suppression must indeed be very potent, active at

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**Table 1. Non-E-Rosette Fcγ' Cell Suppression of GM-CFC**

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<th>Experiment</th>
<th>LDNANOT: Fcγ' Marrow</th>
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<td>320†</td>
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*Mean ± SE of 3 replicates.
†Mean of 2 replicates.
possibly 2500 cells per culture. The nature of a T+ Fcγ' cell is further complicated by recent cell marker studies with monoclonal antibodies. Some feel this population may represent a monocyte cell. Specific functions such as natural killer activity have been assigned to this subpopulation. Until we separate T cells with monoclonal antibodies reactive to T cells before Fcγ' separations, we should conclude that E rather than T cells can suppress GM-CFC growth.

An interesting finding was that both marrow and blood T+ Fcγ' cells also equally suppressed GM-CFC growth. This marrow population did not contain monocytes (by nonspecific esterase assay) but contained an equal proportion of early myeloid forms (promyelocytes, myelocytes, and metamyelocytes) and lymphoid-like cells. This marrow cell population is similar to the cell described by Broxmeyer in leukemic states as releasing a factor called leukemic inhibitory activity. This factor was released only by leukemic cells that were nonadherent T- (NAT-) without complement receptors but Fcγ'. Broxmeyer also described another factor from neutropenic patients with different biologic inhibitory properties and released from marrow NAT populations. We are presently investigating whether soluble factors can reproduce this suppression. Most interesting is that a blood nonmonocytic T- Fcγ' was the most active cell at suppression. This same population is well known to be enriched for NK cell activity. We would not suggest that these are NK cells, as inhibition is not enhanced by cell contact (unpublished observations) and no activity was demonstrated in these experiments in peripheral blood T+ Fcγ' fractions, another fraction enriched in NK cell activity. This study nevertheless shows that Fcγ' cells from normal human marrow and blood suppress autologous GM-CFC growth without mitogen activation.

REFERENCES


Cells with Fc gamma receptors from normal donors suppress granulocytic macrophage colony formation

G Spitzer and DS Verma