

Immunoregulatory T Cell Function in Multiple Myeloma

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ABSTRACT Multiple myeloma is a malignancy characterized by uncontrolled monoclonal B cell differentiation and immunoglobulin production. In most instances, there is concomitant reduction in polyclonal differentiation and immunoglobulin synthesis both in vivo and in vitro. In in vitro pokeweed mitogen-induced B cell differentiation assays, proliferation and polyclonal immunoglobulin secretion optimally requires T cell help and can be inhibited both by monocytes and suppressor T cells. Helper function and monocyte-mediated suppression are relatively radio-resistant whereas T suppressor function is sensitive to 2,000 rad x-irradiation. We have examined myeloma T cell subset function in this assay using recombinations of isolated patient and normal B cells, T cells, and T cell subsets. Monocytes were removed by a carbonyl iron ingestion technique, normal and myeloma T cells were fractionated on the basis of Fc receptors for immunoglobulin (Ig)G ($T\gamma$) or IgM ($T\mu$ or $T\text{non-}\gamma$), and proliferation and IgG secretion after co-culture determined by [^3H]thymidine incorporation and radioimmunoassay, respectively. Myeloma B cells demonstrate quantitatively and qualitatively normal blastogenic responses and are appropriately regulated by either autologous or allogeneic T helper and suppressor subsets. Despite normal proliferation, however, myeloma B cells remain deficient in subsequent differentiation and immunoglobulin secretion even when co-cultured in the absence of monocytes or suppressor T cells and the presence of normal helper cells. Myeloma T cell populations, in contrast, are entirely normal in helper capacity over a range of T:B ratios but are markedly deficient in radiosensitive and concanavalin A-induced suppressor activity. T suppressor cell dysfunction in multiple myeloma is apparently due to a deficit in the $T\text{non-}\gamma$ suppressor subset, whereas $T\gamma$ cells, although proportionately reduced, are functionally normal. This unique T suppressor deficit reflects the heterogeneity of suppressor mechanisms in this disease and may represent a compensatory

response to the monoclonal proliferation or the involvement of regulatory T cells in the pathogenesis of the malignancy.

INTRODUCTION

Multiple myeloma (MM)¹ is clinically defined as a malignant plasma cell proliferation resulting in bone marrow replacement, paraprotein production, osteolytic bone lesions, decreased polyclonal immunoglobulin (Ig) synthesis, and a concomitant increase in susceptibility to certain bacterial pathogens (1-3). The immunodeficiency of myeloma is distinct from that of other malignancies in that both myeloma patients and mice bearing plasmacytomas exhibit impaired primary responses to antigenic challenge, whereas secondary responses as well as delayed hypersensitivity are less severely affected (1, 3-7). A variety of hypotheses have been suggested to explain impaired host polyclonal Ig synthesis in both multiple myeloma and murine plasmacytomas including the alteration of antigen-binding cells by infective RNA subunits from malignant plasma cells (8), the influence of mitotic inhibitors or chalone that block polyclonal B cell differentiation (9), and the presence of circulating immunosuppressive factors produced by murine plasmacytoma cells (10). In contrast to studies attributing the depressed primary responses and Ig levels to an immunosuppressive product of the expanding population of tumor cells, Broder et al. (11) originally suggested that a potential abnormality of host immunoregulatory cells might be involved in the immunodeficiency of myeloma in man (11). They demonstrated suppressor activity by unfractionated myeloma peripheral leukocytes that was mediated primarily by phagocytic mononuclear cells and not by T lymphocytes. Similar adherent cell suppressor mechanisms have been demonstrated in the murine plasmacytoma model (5, 12) and in addition an augmenting role for tumor cell factors on macrophage-

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¹Abbreviations used in this paper: BRBC, bovine erythrocytes; FCS, fetal calf serum; MM, multiple myeloma; n, normal cells; PWM, pokeweed mitogen; RFC, rosette-forming cells; sIg, surface immunoglobulin; SRBC, sheep RBC.

mediated suppression has been suggested. Knapp and Baumgartner (13), however, demonstrated that adherent cell suppressor activity is present in healthy individuals as well as myeloma patients, suggesting that monocyte/macrophage-mediated suppression of B cell differentiation is neither a disease-specific phenomenon nor an explanation of decreased *in vivo* or *in vitro* polyclonal Ig synthesis in myeloma. Paglieroni and MacKenzie (14), in addition, have shown that a significant amount of suppressor activity in myeloma patients is present in a nonadherent lymphoid subset bearing Fc-receptors that does not appear to be thymus-derived as defined by the presence of receptors for sheep erythrocytes (14, 15). This suppressor activity can be demonstrated for antigen-induced blast transformation and autologous or allogeneic B cell differentiation as well as on mixed lymphocyte responses by allogeneic normal T cells. We have extended these studies of Ig-producing deficits in myeloma by examining the functional regulatory capacity of MM T γ and T non- γ subsets on patient and normal B cell differentiation. The data presented here confirm Paglieroni and MacKenzie's observation (14, 15) that myeloma B cell differentiation is markedly impaired even in the absence of suppressor T cells or monocytes. In addition, new data presented here demonstrate: (a) MM B cell differentiation cannot be augmented in the presence of either myeloma or normal T helper subsets, (b) T helper activity in these patients is nonetheless functionally intact over a range of T:B ratios, and (c) unlike the leukemic cell population in most patients with chronic lymphocytic leukemia, the early stage of peripheral B cell differentiation in MM, T cell-regulated B cell proliferation, is virtually normal, whereas (d) a specific T suppressor abnormality can be identified in these patients for T cell-regulated IgG secretion. The suppressor deficit in multiple myeloma is further shown to be confined to the T non- γ suppressor cell subset that demonstrated sensitivity to 2,000 rad x-irradiation and is inducible in the presence of concanavalin A.

METHODS

Patient population. 23 different healthy volunteer age-matched donors who were receiving no medication were used as controls. A total of 21 different patients with MM between 48 and 63 yr of age were included in this study. The diagnosis of multiple myeloma was confirmed by the presence of at least two of the following criteria: (a) plasma cells constituting >20% of the total nucleated cell population of aspirated bone marrow without other known causes; (b) a monoclonal serum or urine gamma globulin peak on immunoelectrophoresis in combination with reduction in other serum Ig, and (c) osteolytic lesions in the skull, vertebral column, or pelvis. Of the 21 patients examined, 14 had not received prior therapy and were newly diagnosed. The remaining seven patients had previously received intermittent therapy with alkylating agents and corticosteroids, however

these patients were sampled at least 6–8 wk following completion of their last drug cycle or were off chemotherapy altogether. 18 of the myeloma patients examined had IgG components and three had IgA; none had macroglobulinemia or light chain diseases.

Isolation of lymphocyte populations. Peripheral leukocyte populations were obtained from buffy coats separated either by leukopheresis of both patients and normal volunteers with a Haemonetics model 30 Cell Separator (Haemonetics Corp., Natick, Mass.) or by gravity sedimentation of 500 ml of citrated blood from some volunteer donors. No differences in surface markers or *in vitro* functional capacity of lymphocytes obtained by either method were apparent in our assays. Informed consent was routinely required before patients were entered into this study. Phagocytic mononuclear cells were depleted by diluting each buffy coat 1:2 with medium containing carbonyl iron (Technicon Instrument Co., Tarrytown, N. Y.) in 50-ml aliquots, and incubating the mixture for 60 min with gentle continuous agitation. Each leukocyte population was then layered in 10-ml aliquots on 3 ml Ficoll-Hypaque density gradients (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), in 15-ml siliconized glass tubes and centrifuged 40 min at 400 g. The interface layer was resuspended in RPMI 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) and washed two times. Viability of the purified lymphocyte populations was routinely >95% and monocyte contamination was always <2% by non-specific esterase staining and latex bead ingestion.

E rosette positive (E⁺) and negative (E⁻) lymphocyte populations were obtained by rosetting with sheep erythrocytes (SRBC) that had been treated with 2-aminoethylisothiuronium bromide according to the method of Kaplan and Clark (16). 2-Aminoethylisothiuronium bromide-treated sheep erythrocytes were washed twice in RPMI 1640 containing 10% fetal calf serum (FCS) and antibiotics, and mixed with lymphocyte suspensions at a ratio of 50 SRBC:1 lymphocyte in 15-ml conical siliconized glass tubes. These mixtures were centrifuged for 8 min at 200 g and incubated 18 h at 4°C. The pellets were resuspended gently, rosette-forming cells (RFC) were enumerated, and the E⁺ population sedimented on Ficoll-Hypaque at 400 g for 40 min. SRBC were removed by brief hypotonic lysis and the pellets resuspended in 3% saline to isotonicity. E⁺ and E⁻ populations were washed twice in supplemented RPMI 1640 and kept at 4°C. Purity of these and other populations were determined by enumerating surface Ig-positive (sIg⁺) cells with rabbit anti-human F(ab')₂-coupled bovine erythrocytes (BRBC, Gibco Laboratories) according to the method of Gold and Fudenberg (17) as previously described (18). Complement receptor-positive lymphocytes were detected by a similar rosetting assay using BRBC sensitized with anti-BRBC IgG and which had been incubated with a 1:10 dilution of fresh human serum previously absorbed at 4°C with BRBC.

Separation of T γ and T non- γ subpopulations. EA.G⁺ (T γ) cells were isolated by positive selection with BRBC coated with the IgG fraction of rabbit anti-BRBC antibody (N. L. Cappel Laboratories, Cochranville, Pa.). This antibody was screened for titer and class specificity and further purified on Sephadex G-150 columns if necessary. T γ RFC were prepared by a modification of the technique of Moretta et al. (19). Briefly, washed BRBC were incubated with a 1:70 dilution of anti-BRBC IgG for 30 min at 37°C in normal saline with occasional mixing, washed twice in saline, and stored at 4°C for use within 4 d. EA.G⁺ RFC were prepared by a 2-h incubation of the E⁺ population with IgG-coated BRBC at 150 BRBC:1 lymphocyte in RPMI 1640 with 10% FCS. The rosettes were resuspended with extremely gentle agitation, layered on Ficoll-Hypaque gradients, and centrifuged for 15

min at 200 g. EA.M⁺ RFC (T μ) were enumerated in an analogous manner using an 18-h incubation at 37°C with BRBC coated with a 1:70 dilution of rabbit anti-BRBC IgM antibodies (N. L. Cappel Laboratories) purified as necessary.

Co-culture methods. IgG produced by isolated subpopulations and in co-culture experiments was determined by a modification of the technique described by Gailani et al. (20). Lymphocyte populations were adjusted to a final concentration of 2×10^6 cells per ml in RPMI 1640 supplemented with glutamine, 0.1 ng/ml gentamicin and previously screened, heat-inactivated 10% FCS. The cell suspensions were incubated in flat-bottomed microtiter plates (Linbro Chemical Co., Hamden, Conn.) for 7 d at 37°C in a humidified 5% CO₂ atmosphere. In co-culture experiments, ratios of B:T cells were maintained at 1:2 while T cell subpopulations were recombined in equal ratios unless otherwise noted such as in the titration experiments. The total well volume was 350 μ l. In some experiments, T cells were irradiated with 2,000 rad x-irradiation from a General Electric Maxitron 250 x-ray machine (General Electric Co., Schenectady, N. Y.) using 0.25-mm Cu and 1-mm Al filters. Proliferation responses were determined on day 3 of culture following a 6-h pulse with 2 μ Ci/well of tritiated thymidine (TdR) (sp act 6.7 Ci/mmol) and subsequent harvesting of the entire contents of the well for liquid scintillation counting. Radioimmunoassay was performed on day 7 by a double antibody technique using 200 μ l of supernate from each well following centrifugation of the plate. Affinity-column purified human IgG, previously shown to be specific by gel double diffusion and immunoelectrophoresis, was labeled with 1 mCi of ¹²⁵I using a chloramine-T method. The dilution of anti-IgG that bound 60% of a suitable amount of labeled IgG was determined. Anti-IgG was prepared by multiple rabbit immunizations with purified IgG protein followed by affinity column purification of the antibody. Antigen-antibody dilution curves were constructed for an antigen range of 1 to 30 ng and a 1:8,000 dilution of antiserum. The amount of goat anti-rabbit Ig added to precipitate the rabbit Ig was twice the equivalence amount. From 10 to 100 μ l of culture supernate or standard was added to 20 μ l of anti-IgG for 1 h at 37°C in 10 \times 75-mm plastic Falcon tubes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) after which 10 μ l of goat anti-rabbit Ig was added and the mixture incubated for 1 h at 37°C. After overnight incubation at 4°C, 1.0 ml of 0.01 M Tris, pH 7.0–7.4, was added to each tube. The tubes were centrifuged at 4°C and 2,000 g for 20 min, after which the supernate was discarded, and the pellet counted. Standards were prepared in 0.01 M Tris with 1% bovine serum albumin. The binding of labeled IgG could be shown to be >90% specifically inhibited by cold IgG.

Con A-induced suppression of IgG secretion. T lymphocyte populations or T cell subsets from both patients and normal donors were activated in the presence of 5 μ g/ml concanavalin A (Con A) (Calbiochem Behring Corp., American Hoechst Corp., San Diego, Calif.) for 48 h in RPMI 1640 supplemented with 10% FCS, 200 mM L-glutamine, antibiotics, and 25 mM Hepes buffer at a concentration of 4×10^6 cells/ml. Untreated control cells were cultured in an identical fashion without Con A. Following activation, cultured T cells were washed three times, adjusted to a concentration of 2×10^6 cells/ml, and incubated in standard culture media for 20 min in the presence of 25 μ g/ml mitomycin-C (Bristol Laboratories, Syracuse, N. Y.). These activated T cells and subsets were subsequently washed four times, readjusted to 2×10^6 cells/ml and added to normal allogeneic irradiated T and unirradiated T co-cultures to determine Con A-induced suppression of maximal IgG secretion. In preliminary experiments, 0.3 M α -methyl-D-mannoside (Sigma Chemical

Co., St. Louis, Mo.) was included in the wash, but was found not to influence the results significantly and reported experiments were performed in the absence of α -methyl-mannoside. The percent suppression of total IgG secretion was calculated according to the following formula:

percent suppression

$$= \frac{\text{IgG secretion by B + Tx + cultured T} - \text{IgG secretion by B + Tx + cultured T Con A}}{\text{IgG Secretion by B + Tx + cultured T}} \times 100.$$

where B + Tx co-cultures were performed using normal autologous cell populations and allogeneic cultured T cells as controls. T Con A cells consisted of allogeneic normal or patient T cells and subsets incubated 48 h with Con A. Total co-cultures contained 1×10^5 cells of each subpopulation.

Statistical analysis. Data are presented as either ranges or means \pm SEM of the total patient and normal donor populations for both [³H]thymidine incorporation and IgG production. Individual experiments were performed with paired patient and normal samples in triplicate in each instance, and the individual means were used to calculate the group means as presented. The statistical significance of the difference between mean values in the various groups was determined by the student's *t* test and for individual pairs by the paired *t* test. A *P* value of <0.05 was considered to represent a significant result and is so indicated in the figures and tables.

RESULTS

Surface markers of lymphocyte subsets. Myeloma lymphocyte populations were readily separable into E⁺ and E[−] populations with at least 95% purity in every instance by repeat surface marker analysis (Table I). The E[−] population was greatly enriched for sIg-bearing cells and cells bearing complement receptors. In both patients and normal individuals, an average of 70% of this population is identifiable as B lymphocytes by the presence of sIg. The remainder of the E[−] population from normal donors consists of a very small proportion of contaminating E⁺ T cells (3–5%) and a null population which is heterogeneous for Ia, complement and Fc receptors, but highly active in two in vitro assays: antibody-dependent cellular cytotoxicity and natural killing (18, 21). γ FcR⁺ and μ FcR⁺ subsets of T lymphocytes could also be easily identified and the Ty fraction readily separated from both myeloma and normal T lymphocyte populations. The proportions of total T cells bearing receptors for either IgG or IgM were equally variable in patients and normal individuals and ~90–95% of the total T population was found to bear Fc receptors for one or the other class of Ig. Repeat Fc-rosetting following positive selection demonstrated enrichment to at least 65–90% of cells bearing the Fc receptor specifically isolated, whereas <3% of either subset bore the nonselected Fc receptor. Thus both the Ty and T μ subset could be isolated with >95% purity by negative criteria although masking of the receptors during the separation apparently prevents repeat rosetting of a proportion of

TABLE I
Surface Characteristics of Normal and Myeloma Lymphocyte Subsets

Lymphocyte subset	Percent E ⁺	Percent sIg ⁺	Percent EAC ⁺	Percent γ FcR ⁺	Percent μ FcR ⁺
Normal donors (n = 14)					
Unfractionated PBL	63-80	5-18	7-28	—	—
E ⁻ (B cells + null cells)	3-5	68-92	39-76	—	—
E ⁺ (T cells)	94-99	0-3	2-6	16-24*	17-62
				Mean = 19.3 \pm 2.3 SD	
EA.G ⁺ (T γ)	92-98	<2	<2	67-89†	<2
EA.G ⁻ (T non- γ)	94-99	<2	2-4	0-3	54-82†
Myeloma patients (n = 13)					
Unfractionated PBL	64-80	7-21	5-22	—	—
E ⁻ (B cells + null cells)	4	64-87	40-82	—	—
E ⁺ (T cells)	95-99	0-3	<2	3-15*	26-60
				Mean = 10.9 \pm 3.9 SD	
EA.G ⁺ (T γ)	94-98	<2	<2	63-88†	<2
EA.G ⁻ (T non- γ)	95-99	<2	<2	<2	54-84†

* The difference between mean percent EA.G⁺ cell numbers in myeloma and normal is significant by both Student *t* and paired *t* tests (*P* < 0.05).

† Determined by a second rosetting. Purity of separated rosette-forming cells after Ficoll-Hypaque gradient separation and prior to BRBC lysis was always >95%.
PBL, peripheral blood leukocytes.

each subset following isolation. A minor but consistent difference in numbers of T γ cells in patient T populations compared to normal donor T cells could be identified. Myeloma T γ populations were proportionately lower (mean 10.9 \pm 3.9% SD) than normal T γ populations (mean 19.3 \pm 2.3% SD; *P* < 0.05). All other surface markers, including μ FcR, were essentially identical in myeloma and normal lymphocyte populations. Despite high concentrations of M components and a concomitant reduction in serum polyclonal Ig in our patient population, there were no apparent differences in absolute numbers or proportions of circulating sIg⁺ cells in patients compared to normal donors.

T cell-regulated IgG production by normal and myeloma B cells. The in vitro production of IgG by isolated normal and myeloma lymphocyte populations after pokeweed mitogen (PWM) stimulation was determined in the absence of monocytes (<2%) for 14 normal donors and 13 myeloma patients. IgG secretion after a 7-d incubation period is expressed as nanograms IgG per milliliter, which was equivalent in our culture system to nanograms/3 \times 10⁵ total lymphocytes or nanograms per liter \times 10⁵ B cells cultured (Table II).

Isolated T and B populations from normal donors and patients produced only minimal amounts of IgG while co-culture of normal T and B populations produced ~1,300 ng/ml. In contrast, monocyte-depleted myeloma T and B co-cultures were significantly depressed

(*P* < 0.005) in their ability to produce IgG. In an initial attempt to determine the role of myeloma T cell regulation of polyclonal Ig synthesis, we cultured autologous B and T cells after prior x-irradiation of the total T populations. Moderate doses (1-2,000 rad) x-irradiation have been previously demonstrated to selectively abrogate polyclonal suppressor T cell function in a similar assay system (22), while adherent suppressor cells are relatively more radioresistant (13).

TABLE II
T Cell-Regulated IgG Production by Peripheral B Cells from Normal Donors and Myeloma Patients

Composition of cells cultured (PWM added at 1:200)	IgG secretion (ng/ml/7 days \pm SEM)	
	Normal donors (n = 14)	Myeloma patients (n = 13)
T cells alone	22 \pm 21	25 \pm 14
B cells alone	133 \pm 29	191 \pm 66
B + T	1,295 \pm 120	172 \pm 42
B + Tx	1,987 \pm 317*	236 \pm 66
MM-T + Bn	1,434 \pm 150	
MM-Tx + Bn	1,488 \pm 177	

n, normal. x, 2,000 rad x-irradiation. Ratios of co-culture: B:T = 1 \times 10⁵:2 \times 10⁵.

* The mean IgG production following irradiation of autologous T cells was significantly increased over nonirradiated T cells (*P* < 0.05).

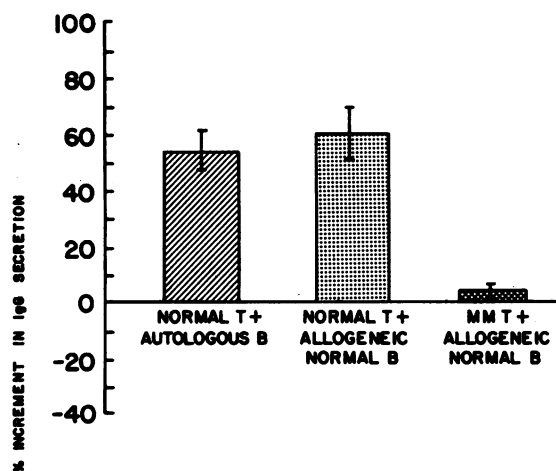


FIGURE 1 Radiosensitive suppressor T cell function in MM: comparison of normal B cell IgG secretion after 7 d in co-culture with untreated and irradiated T cells from normal donors and myeloma patients. Results are expressed as the percent increment in total IgG secreted in co-cultures containing irradiated (2,000 rad) T cells \pm SEM. Culture conditions are described in the text.

IgG production by co-cultures of B plus irradiated autologous T cells from normal donors was significantly increased over nonirradiated T cells ($1,987 \pm 317$ ng vs. $1,295 \pm 120$ ng, $P < 0.05$). Only a very minor and insignificant increment in mean IgG production was observed in co-cultures of myeloma B cells and irradiated myeloma T cells (236 ± 66 ng vs. 172 ± 42 ng, $P > 0.10$) and in a number of patients a decrease in IgG

secretion could be demonstrated after x-irradiation of co-cultured T cells. In an effort to assess the helper and suppressor capacity of myeloma T cells, co-cultures of patient T and allogeneic normal B cells were compared with and without x-irradiation (Table II). Myeloma T cells were able to help normal B cell IgG production virtually as well as autologous T cells; however, x-irradiation of the myeloma T population did not result in any increment in IgG production by normal B cells as was consistently demonstrated with irradiated normal T cells. This difference is not simply the result of allogeneic effects since, as shown in Fig. 1, there are significant increases in IgG production with prior T cell x-irradiation whether the differentiating B cell population is autologous or allogeneic. In order to exclude the possibility that a more subtle deficiency in T helper activity might be involved in MM, we also examined T cell help for Ig secretion using serial dilutions of normal and patient T cells co-cultured with a constant 1×10^5 normal B cells. In the three separate patients and normal controls shown in Fig. 2, no significant difference in helper function could be identified even at T:B ratios of 0.1. The radiation-induced increment in IgG secretion was evident only for normal donor T populations at T:B ratios of 0.5 or greater and was non-existent in these three myeloma patients. Thus, although myeloma T cells are relatively normal in helper function, we were unable to demonstrate the expected radiosensitive suppressor activity by total myeloma T lymphocyte populations.

Immunoregulatory capacity of normal and myeloma T cell subsets on autologous B cell function. This

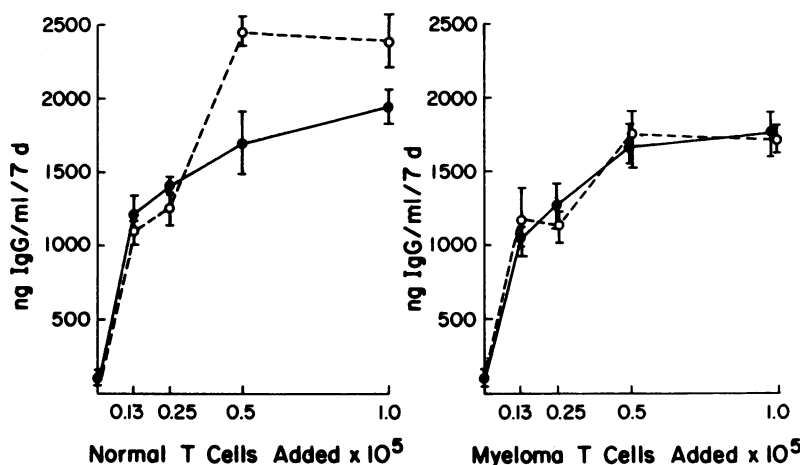


FIGURE 2 Comparison of myeloma and normal donor x-ray-induced increments in IgG secretion with varying T:B ratios. A constant number (1×10^6) of B cells was co-cultured for 7 d with varying numbers of T cells from normal allogeneic donors or myeloma patients. Solid lines represent the addition of unirradiated T cells, whereas dashed lines represent T cells added after 2,000 rad x-irradiation. Results are expressed as means for three individual normal donor and patient co-cultures \pm SEM.

apparent deficiency in radiosensitive T suppressor activity in myeloma T cell populations could result either from the proportional decrease in the T γ subset within the total T cell population or from a functional abnormality of the suppressor subsets. In order to determine which of these possibilities was contributing to the observed suppressor abnormality, we compared isolated T γ and T non- γ cells from myeloma patients in 72-h thymidine incorporation and 7-d B cell differentiation assays. Table III demonstrates the results obtained in 14 healthy subjects and 13 myeloma patients for autologous B and T cell subsets.

As with IgG secretion, isolated B cells from either patients or normal donors exhibited minimal blastogenic responses to PWM. Total T cells responded well to PWM and the T non- γ subset demonstrated an increase in mean thymidine incorporation over unfractionated T cells. T γ cells, on the other hands, were not only poorly responsive to PWM, but were capable of suppressing the blast response of an equal number of T non- γ cells by nearly 50%. Irradiated T cells cultured alone or in the presence of irradiated B cells still demonstrated a small blastogenic response, although significantly below that of B + Tx co-cultures. Aside from an insignificant difference in mean change in

counts per minute by myeloma T and B cell populations compared with normal, there were no qualitative or quantitative differences between patient and normal lymphocytes in blast responsiveness. When T and B cells were co-cultured, thymidine incorporation was more than doubled in both normal and patient populations compared to either population cultured alone. As has been previously described (19) only T non- γ cells provided help for both thymidine incorporation and IgG secretion. The T γ subset did not allow either B cell proliferation or antibody synthesis, and in co-culture with T non- γ and B cells, suppressed both blast responsiveness and Ig secretion. Myeloma patient blast responses appeared to be well within the normal range for B and T cooperative responses, suggesting that myeloma B cells were actively synthesizing DNA; however, the amount of IgG subsequently secreted by patient B cells was markedly depressed even in the complete absence of either T suppressor cells or monocytes.

Although a low to moderate dose (2,000 rad) x-irradiation of the T non- γ subset resulted in a decrease in total thymidine incorporation for B and T co-cultures, a radioresistant increment in helper activity for Ig secretion was demonstrable for this subset from

TABLE III
Immunoregulatory Capacity of Normal and Myeloma T Cell Subsets

Composition of co-culture	Normal donors, n = 14		Myeloma patients, n = 13	
PWM added at 1:200	[³ H]TdR incorporation		[³ H]TdR incorporation	
	Mean Δ CPM \pm SEM*		Mean Δ CPM \pm SEM	
B cells alone	2,543 \pm 1,310		1,864 \pm 1,436	
T cells alone	8,005 \pm 1,361		7,160 \pm 1,431	
T non- γ cells alone	10,114 \pm 2,372		8,904 \pm 1,215	
T cells alone	1,426 \pm 1,838		520 \pm 297	
T non- γ + T γ cells	5,738 \pm 1,321		4,977 \pm 732	
xT alone	1,421 \pm 1,221		1,583 \pm 1,002	
xB + xT	2,883 \pm 1,541		2,290 \pm 1,388	
	[³ H]TdR incorporation Mean Δ CPM \pm SEM	Mean IgG secretion ng/ml per 7 d	[³ H]TdR incorporation Mean Δ CPM \pm SEM	Mean IgG secretion ng/ml per 7 d
B + T	21,010 \pm 2,632	1,295 \pm 120	18,943 \pm 3,458	172 \pm 42
B + xT	9,214 \pm 895	1,987 \pm 317	8,204 \pm 1,280	236 \pm 66
B + T non- γ	24,977 \pm 3,006	1,220 \pm 248	22,338 \pm 3,379	256 \pm 72
B + xT non- γ	13,877 \pm 1,342	1,886 \pm 252	11,923 \pm 2,301	244 \pm 61
B + T γ	3,384 \pm 1,626	231 \pm 52	3,139 \pm 1,200	252 \pm 17
B + xT γ	2,104 \pm 595	163 \pm 45	1,895 \pm 601	192 \pm 22
B + (T non- γ + T γ)	18,955 \pm 2,894	685 \pm 150	16,408 \pm 2,444	260 \pm 41

x = 2000 rad x-irradiation.

Ratios of co-culture: B:T = 1×10^5 : 2×10^5 , T non- γ :T γ = 1×10^5 : 1×10^5 .

* [³H]tritiated thymidine (TdR) incorporation expressed as counts per minute per culture well.

TABLE IV
Radiosensitivity of Myeloma and Normal T Helper and Suppressor Subset Function

Composition of co-culture			Percent change in IgG secretion	
Normal B + normal T subsets	[³ H]TdR incorporation Mean ΔCPM	Mean IgG secretion ng/ml per 7 d	Percent enhancement after x-irradiation	
10 ⁵ B + 2 × 10 ⁵ T non-γ	51,969 ± 3,306*	1,136 ± 192	—	
10 ⁵ B + 2 × 10 ⁵ xT non-γ	17,382 ± 1,492	1,760 ± 204	+54.9%	
			Percent suppression by T _γ cells	
10 ⁵ B + 10 ⁵ T non-γ	+1 × 10 ⁵ xT _γ	44,171 ± 3,100	1,040 ± 166	8.4%
	+5 × 10 ⁴ T _γ	47,940 ± 2,809	880 ± 81	22.5%
	+1 × 10 ⁵ T _γ	31,297 ± 1,486	650 ± 52	42.8%
	+2 × 10 ⁵ T _γ	24,537 ± 2,108	390 ± 63	65.7%
Normal B + myeloma T subsets			Percent enhancement after x-irradiation	
10 ⁵ B + 2 × 10 ⁵ T non-γ	41,140 ± 3,363	1,312 ± 123	—	
10 ⁵ B + 2 × 10 ⁵ xT non-γ	13,342 ± 1,892	1,105 ± 128	-15.8%	
Normal B + myeloma T subsets			Percent suppression by T _γ cells	
10 ⁵ B + 10 ⁵ T non-γ	+1 × 10 ⁵ xT _γ	43,457 ± 3,211	1,255 ± 183	6.8%
	+5 × 10 ⁴ T _γ	36,663 ± 2,776	1,040 ± 104	20.7%
	+1 × 10 ⁵ T _γ	29,595 ± 1,764	720 ± 120	45.1%
	+2 × 10 ⁵ T _γ	25,172 ± 1,285	520 ± 86	60.4%

x, 2,000 rad x-irradiation.

* Results expressed as means of four separate experiments ± SEM.

[³H]tritiated thymidine (TdR) incorporation expressed as counts per minute per culture well.

normal donors. Patient B lymphocytes, however, while demonstrating the normal cooperative blast responsiveness in co-culture with autologous irradiated T or T non-γ cells, remained unable to differentiate into plasma cells, even in the presence of irradiated T non-γ cells. X-irradiation of the normal or MM T_γ subset did not substantially improve its complete lack of helper activity in either the blast or Ig secretion assay, confirming the absence of cells with radioresistant helper function within this subpopulation.

Immunoregulatory capacity of myeloma T cell subsets on normal B cell function. As a next step in analyzing T helper and suppressor functional capacity in MM, we examined the ability of isolated T_γ and T non-γ subsets from myeloma patients to augment or depress normal allogeneic B cell differentiation. Results obtained in four myeloma patients and in four normal controls are shown in Table IV. The normal T non-γ subset provides helper function for autologous B cell DNA synthesis and prior irradiation of this subset results in >50% increment in IgG secretion. T_γ cells, in contrast, suppress both blast responsiveness

and IgG secretion of co-cultured B and T non-γ cells in a dose-dependent fashion. Myeloma T non-γ cells co-cultured with allogeneic normal B cells provide radioresistant helper activity that is equal to autologous T cells. Similarly, myeloma T_γ cells can suppress normal B cell responses in the presence of the myeloma T non-γ subset and this suppressor activity is radio-sensitive to 2,000 rad. A marked distinction between myeloma and normal T helper function is demonstrated, however, by irradiation of the T non-γ population. As previously described for total myeloma T cell populations, x-irradiation of the T non-γ subset also fails to produce the normal increment in IgG secretion and, in these four patients, resulted in a small decrease in mean IgG production. These results support those obtained with unfractionated T and B populations in the 13 original patients and suggest that, while T helper and T_γ suppressor cell function are intact, there is an additional radiosensitive T suppressor subset that is functionally deficient in multiple myeloma and that lacks Fc receptors for IgG. In order to confirm that patients with multiple myeloma are de-

TABLE V
Con A-inducible Suppressor Activity by Normal and Myeloma T Cell Subsets

Composition of co-culture		Mean IgG secretion ng/ml per 7 d	Percent suppression of IgG synthesis %
Normal donor cells	Con A-induced suppressor population		
1 × 10 ⁵ B + 1 × 10 ⁵ Tx	+1 × 10 ⁵ nT Controls cultured without Con A	2,130±384	—
	+1 × 10 ⁵ nT	388±126	82
	+1 × 10 ⁵ nT non-γ	427±183	80
	+1 × 10 ⁵ nTx	2,359±366	0
	+1 × 10 ⁵ MM-T	1,978±236	8
	+1 × 10 ⁵ MM-T non-γ	1,921±192	10
	+1 × 10 ⁵ MM-Tx	2,077±243	2

x, 2,000 rad x-irradiation. Where indicated, suppressor cell populations were irradiated prior to Con A activation. Results expressed as means of four separate experiments±SEM. Suppressor cells were incubated 48 h in the presence of 5 μg/ml Con A and subsequently with 25 μg/ml mitomycin-C as described in the text.

ficient in radiosensitive T non-γ subset function, we have also examined the ability of total patient T and T non-γ cells to generate suppressor activity for IgG synthesis in the presence of Con A as shown in Table V. Co-cultures of normal B and irradiated normal T cells were used as an indicator system and total T cells and the T non-γ subset from four patients and four normal donors were activated in the presence of 5 μg/ml Con A for 48 h and subsequently treated with 25 μg/ml mitomycin-C. When Con A-activated normal T or normal T non-γ cells were added to B + Tx co-cultures on day 0, IgG secretion was inhibited by 80%. In contrast, MM-T or MM-T non-γ cells suppressed subsequent IgG secretion by only 10% or less. Irradiation of normal T cells with 2,000 rad prior to Con A activation abrogated the mitogen-induced generation of suppressor activity confirming the radiosensitivity of this suppressor population's activity. MM-T cells from these four patients were unaffected by a 48-h culture in the absence of Con A and lacked significant suppressor activity for Ig secretion.

DISCUSSION

A variety of in vitro immune functional abnormalities have been described in both murine and human systems in an effort to explain the inability of myeloma B cells to differentiate into plasma cells capable of secreting Ig, either in response to specific antigens or nonspecific mitogens. Using a murine plasmacytoma model, Zolla et al. (4, 6) demonstrated that although cells with antigen receptors are apparently normal in number and function, both primary and secondary immune responses are depressed in direct correlation

with tumor size. Using a PWM-driven co-culture assay analogous to that described here, Broder et al. (11) demonstrated decreased Ig synthesis by normal donor lymphocytes in the presence of adherent cells but not of T cells from myeloma patients. They suggested, therefore, that impaired Ig synthesis by patient B cells was secondary to increased activity by a monocyte suppressor population. Normal donor adherent cells may, however, also suppress B cell differentiation to an equal degree in this assay (13). Paglieroni and MacKenzie (14, 15) demonstrated a significant amount of suppressor activity in myeloma lymphocyte populations that were essentially free of adherent or phagocytic cells. In their studies the suppressor activity appeared to be mediated by lymphocytes bearing Fc receptors that were present following carbonyl-iron depletion of phagocytic/adherent cells. Although they suggested that this suppressor subset may represent a nonphagocytic monocytoïd cell such as that described by Hayward and Greaves (23), other possible candidates include FcR⁺ null lymphocytes or T_γ lymphocytes, which have been suggested to represent the same or similar cell subsets at different stages of differentiation or function (21, 24). The suppression of MM B cell differentiation appears distinct from the depressed Ig secretion seen in vivo and in vitro with leukemic B cell populations from most patients with chronic lymphocytic leukemia. In those patients neither B cell proliferation nor subsequent Ig secretion occur unless patients are in remission (25) indicating an intrinsic functional deficit of the circulating leukemic population. In contrast, our data indicate that the peripheral B cell population of myeloma patients can proliferate in response to PWM and in the

presence of autologous irradiated T cells but subsequently fails to differentiate and secrete Ig. This further supports the notion of a functional increase in suppressor activity (14, 15), which appears to operate at a later point in B cell differentiation than DNA synthesis.

The results presented here argue strongly against an abnormality of either $T\gamma$ suppressor activity or T non- γ helper function in MM. Both T non- γ helper and $T\gamma$ suppressor function were intact in untreated patients despite the inability of autologous patient E^- cells (which contained FcR^+ null cells as well as sIg^+ cells) to differentiate into plasma cells. In an effort to examine myeloma suppressor function by a parameter independent of $T\gamma$ subset interaction with antigen-antibody complexes, we have also used the functional sensitivity of T suppressor activity to low-dose x-irradiation and Con A-induction of suppressor activity. Radioresistance of T cell help and sensitivity of T suppressor function to low doses of x-irradiation has been described for total T cells (22) as well as fractionated $T\gamma$ cells (19). The failure of the irradiated patient total T population to demonstrate the expected increment in help suggested either a functional or proportional $T\gamma$ deficit in MM. In the experiments described here, the T non- γ subset from normal donors demonstrated an x-ray induced increment in IgG secretion equal to total irradiated T cells. Although this might possibly be explained by a failure to totally deplete T cells with IgG receptors, it seems highly improbable that the 2–3% of contaminating $T\gamma$ cells detected after separation could account for the observed 50% increment in helper activity following irradiation or for Con A-inducible suppressor activity within the T non- γ subset. The presence of radiosensitive suppressor cells in the T non- γ fraction has been previously described (26) and although both the $T\gamma$ and T non- γ subsets contain suppressor activity, the Con A-inducible T suppressor cell defined phenotypically with monoclonal antibodies lacks Fc receptors for IgG (24).

It appears more likely, therefore, that help and suppression cannot be ascribed to mutually exclusive T cell subsets based on the cellular expression of a particular class of Fc receptor. Pichler et al. (27) have previously demonstrated the modulation of $T\gamma$ to $T\mu$ cells following interaction with immune complexes and suggested that expression of a given receptor might reflect an activational state rather than a unique functional subset analogous to murine T cell subsets defined by alloantisera (28, 29). Our data are consistent with this hypothesis and provide evidence that human T suppressor cells are indeed heterogeneous with respect to surface Fc receptors for IgG and IgM.

Sensitivity to both low-dose irradiation and to corticosteroids in vitro appears to be a more uniform characteristic of human suppressor T cells in B cell differentiation assays than does the presence of receptors for IgG (22, 30, 31) suggesting the necessity for DNA synthesis as a prerequisite to suppressor function by at least some T cell subsets. Similar results have been obtained in murine T suppressor experiments (32) although this conclusion remains controversial, perhaps because of the wide variety of assays used in measuring suppression. Our finding that total myeloma T populations as well as the MM T non- γ subset fail to demonstrate an x-ray induced increment in help or Con A-inducible suppressor activity supports the conclusion that the normal T non- γ subpopulation contains prosuppressor T cells that are functionally deficient in myeloma patients. Although a reduction in total T suppressor activity could result from a proportional decrease in circulating $T\gamma$ cells, this would not explain the distinction between the normal and patient T non- γ subpopulations. The dose-dependent suppressor effects obtained with the myeloma $T\gamma$ subset on allogeneic normal B cells clearly demonstrate that this suppressor deficiency is not due to a functional abnormality in those T cells with Fc-IgG receptors, although the proportional decrease in $T\gamma$ cells could be contributory to the absence of irradiation-induced increments of helper activity in total myeloma T populations. Other possible mechanisms that might play a role in explaining the absence of the radiation-induced increment in IgG secretion described also appear unlikely. The arguments that myeloma T helper cells are more radiosensitive or that MM-T non- γ suppressors more radioresistant than their normal counterparts appear to be countered by our demonstration that Con A-inducible suppressor activity is also deficient in myeloma. Moreover, the results cannot, apparently, be explained in postulating a quantitative decrease in patient helper T cells leading to a relatively greater effect of x-irradiation on MM-T cell help than on normal T cells and thus to an absence of the expected augmentation of IgG synthesis. Since patient T cell helper activity was equivalent to normal help even at very low T:B cell ratios, it would appear that T helper cells are present in equal proportions in both myeloma and normal T cell populations.

Our findings are consistent with previous descriptions of immunoregulatory cell function in MM (11, 13, 14, 15) and provide additional data delineating the function of helper and suppressor T cells in this malignancy. T helper function for polyclonal Ig secretion is clearly intact despite the suppressed B cell function. The suppressor activity of the $T\gamma$ subset is likewise normal despite a minor decrease in the numbers of circulating $T\gamma$ cells. Of interest, however, is

the clear demonstration of radiosensitive suppressor activity by the T non- γ subset, which is proportionally greater than the contribution of suppression by Ty cells in normal individuals and virtually absent in myeloma patients. These results provide functional confirmation for those of Reinherz et al. (24) who have indicated that the normal peripheral T non- γ population contains both Con A-inducible suppressor cells and inducer (helper) T cells as defined phenotypically by monoclonal antibodies to human T cell subsets. For functional regulation of B cell differentiation the Ty subset in both normal individuals and myeloma patients evidently contains suppressor cells that do not require Con A activation. The Ty subset, however, lacks the helper activity of the T non- γ subset. Whether this subset is a monocytoid cell, a true thymus-derived lymphocyte, or an Fc γ ⁺ null cell remains uncertain (21, 23, 24, 33) although its suppressor sensitivity to 2,000 rad x-irradiation makes it functionally more analogous to Con A-inducible T non- γ and Ty suppressors than to adherent cells capable of suppressing B cell differentiation in a similar assay system (13). The explanation for abnormalities of suppressor cell function in MM also remains elusive. The T suppressor dysfunction demonstrated here may represent a normal compensation of the regulatory network in response to increased activity by non-T suppressor populations or, alternatively, the immunopathology of multiple myeloma may actually involve regulatory T cell subsets.

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