Human myeloma cells were purified from bone marrow aspirates from patients with multiple myeloma (MM) by Percoll discontinuous density-gradient centrifugation, E rosette formation and treatment with antiamyelomonocytic antibody (Leu M1) plus complement. Thus, the purified cell fraction consisted of >90% myeloma cells, even when as little as 15% myeloma cells were contained in bone marrow mononuclear cell fraction, determined by morphological and immunologic examinations. With highly purified myeloma cells from 29 patients with IgG type MM, biologic characteristics such as spontaneous proliferation (5H-TdR uptake) and M-protein secretion rate in vitro were evaluated. Both activities varied among patients within stage I and III, and a 5H-TdR uptake of 255-24, 132 cpm/4 x 10^4 cells, and an M-protein secretion rate of 9 to 72 pg/cell/day, respectively, were recorded. However, in each patient, there was no correlation between 5H-TdR uptake and M-protein secretion rate. These results thus suggest that 5H-TdR uptake and M-protein secretion rate of highly purified myeloma cells are independent biologic parameters, not associated with the clinical stages, and the purification of myeloma cells we describe can contribute to further studies on the biologic characteristics and to understanding of the pathophysiology involved in MM.

**MATERIALS AND METHODS**

Patients. BM aspirates were obtained from 29 patients with IgG type MM before or after undergoing treatment. Prior chemotherapy was interrupted at least 1 month before sampling. Clinical staging in patients with MM was done according to the classification of Durie and Salmon. Informed consent was obtained from all patients according to institutional policy.

Preparation of myeloma cells. Heparinized bone marrow aspirates were centrifuged at 550 g for 15 minutes on Ficoll-Hypaque gradient cushion. The cells at the interface were collected (BM mononuclear cells, BMNCs), and fractionated on Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) discontinuous density gradient as described by Dagg and colleagues.

Discontinuous Percoll gradients, each consisting of 2 mL 90%, 60%, 50%, and 45% of Percoll solution, were formed in a 15-mL tube (Corning, NY). Then 1 mL 30% solution suspending the BMNCs (1 x 10^7 cells/mL) and 2 mL 20% solution were layered on the gradient. The tubes were centrifuged at 550 g for 30 minutes at 25°C. Cells banding at interfaces between 60% and 50% (Fr. 3), and 50 and 45% (Fr. 2) were collected. A more enriched fraction of myeloma cells was harvested from these two fractions (usually Fr. 2), and then rosette formation with sheep RBCs (SRBCs) (E rosetting) was performed. Non-E rosetting cells (E- cells) were harvested and treated with 1/5 diluted antiamyelomonocytic monoclonal antibody Leu M1 (Becton Dickinson) plus rabbit complement (Hoechst Behring). After the cells were washed three times with RPMI 1640 medium (Nissui, Japan) supplemented with 10% fetal calf serum (FCS, M.A. Bioproducts, Walkersville, MD), cell viability in this myeloma cell-rich fraction was examined by trypan blue dye exclusion method. Purity of myeloma cells was enumerated morphologically by light microscope (Wright’s stain) and immunologically by direct immunofluorescence.

In vitro proliferation activity. The myeloma cell-rich fraction (>90% myeloma cells, 4 x 10^6 cells/200 µL/well), obtained as

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determined morphologically. This was then confirmed by 92.0% to 98.0% (mean 95.2%) myeloma cells, which were staining for the cytoplasmic immunoglobulin (clg), using cytess, which were removed by shown in Fig 1, for patients I through 3 in whom the percentage of 2, in which the percentage of treatment. Finally, the cell fraction obtained consisted of human IgG (human IgG, normal, TAGO). M-protein standard diamine (OPD) substrate in a presence of H2O2 was then recorded at 405 nm by a plate reader (SLT-Labinstruments, Austria). Concentration of M-protein was determined according to absorbance of standard human IgG (human IgG, normal, TAGO). M-protein secretion activity was defined as follows; concentration of M-protein in the supernatant (µg/mL)/culture days (days) cell number (/mL) \* 1,000 (pg/cell/day).


Statistical analysis. Correlation coefficient between \(^{3}H\)-TdR uptake and M-protein secretion rate in myeloma cells from each patient was assessed by Spearman’s method. The statistical significance in mean values of \(^{3}H\)-TdR uptake and M-protein secretion rate between stage I and III was analyzed by Student’s \(t\) test.

RESULTS

Myeloma cell separation. Myeloma cells from 29 patients were purified by Percoll gradient centrifugation, E rosette formation and treatment with Leu M1, plus complement. Figure 1 shows results of purification in five representative cases. In these cases, BMMNCs obtained by Ficoll-Hypaque density gradient contained 15.0% to 75.0% (mean 40.0%) myeloma cells. Thus, BMMNCs were a suspension of mixed cells consisting of myeloma cells, myeloid cells (mainly myelocytes and metamyelocytes), erythroblasts, lymphocytes, and monocytes. Percoll gradient sedimentation removed a large proportion of monocytes and erythroblasts from BMMNCs. Monocytes did concentrate at a low density fraction, an interface between 30% and 45% Percoll (Percoll Fr. 1). On the contrary, erythroblasts were separated at a high density fraction, 60% and 90% (Fr. 4). A larger proportion of myeloma cells was concentrated at the interface between 45% and 50% Percoll (Fr. 2), and the smaller one was at the interface between 50% and 60% (Fr. 3). Thereafter, Percoll Fr. 2, in which the percentage of myeloma cells was between 47.5% and 87.0% (mean 69.7%), was collected and E rosette formation was performed. The remaining cells, other than myeloma cells, following the E rosetting, were myeloid cells and a small number of monocytes, which were removed by Leu M1, plus complement treatment. Finally, the cell fraction obtained consisted of 92.0% to 98.0% (mean 95.2%) myeloma cells, which were determined morphologically. This was then confirmed by staining for the cytoplasmic immunoglobulin (clg), using FITC-conjugated anti-human IgG (data not shown). As shown in Fig 1, for patients 1 through 3 in whom the myeloma cell content within BMMNCs was 15%, 21%, and 27%, final purity was 96%, 92%, and 96%, respectively. Thus, the results suggest that when at least ≥15% myeloma cells were contained within BMMNCs fraction, our procedure gave a reproducible purity of >90% for these cells. Myeloma cell recovery in these representative cases ranged from 30.0% to 85.0% (mean 56.4%). In a few other cases with a very low percentage (<5%) of plasma cells in BMMNCs, however, such as in early monoclonal gammapathy (BMG), final purity was <50% (data not shown); thus, these cases were excluded from the present study. Cell viability for the isolated myeloma cells was >85% to 90% in all cases examined.

In vitro proliferation and M-protein secretion activity. Proliferation and M-protein secretion activities in vitro for the purified myeloma cells (with at least 90% purity) obtained from 29 patients were investigated. Proliferation activity was evaluated by an uptake of \(^{3}H\)-TdT pulsed during the last 12 hours in a 48-hour culture. Uptake counts in the first 12 hours of labeling were almost equal or low as compared with the last 12-hour pulse applied in this study (eg, 619 cpm in the first 12-hour labeling v 624 cpm in the last 12-hour labeling: 3,654 v 3,150, 2,880 v 5,791, and 7,722 v 10,047). Therefore, we used the last 12-hour labeling to measure the proliferation activity. The M-protein secretion rate was determined by quantification of the M-protein secreted in culture supernatants during the 48 hours because the amount of secreted M-protein increased in proportion to the culture period, at least within 72 hours (Fig 2). The measurement of proliferation and M-protein secretion was suggested to be reproducible when myeloma cells were purified to at least >90%, since the preliminary experiments
Fig 2. M-protein secretion kinetics in highly purified myeloma cells. Purified myeloma cells from two patients were cultured for an indicated time, and the cumulative amount of secreted M-protein in the culture supernatant was measured by ELISA as described in the Materials and Methods section. The absorbance of the culture medium, which is the background and defined as that at time zero, was subtracted from each measured absorbance.

had shown that values of \(^{3}\text{H}-\text{TdR}\) uptake as well as M-protein secretion rates were almost equal in the same untreated patient at an interval of 1 or 2 weeks (data not shown).

The association between the potentials of proliferation and M-protein secretion was investigated with purified myeloma cells. As shown in Fig 3, no linear correlation was detected statistically \((n = 29, r = .02, P > .05)\). This result shows that levels of \(^{3}\text{H}-\text{TdR}\) uptake in each myeloma cell were not associated with the M-protein secretion rate of the cell. Moreover, since wide variations were observed in both \(^{3}\text{H}-\text{TdR}\) uptake and M-protein secretion rates among patients, the association with clinical stages was also examined (Fig 4). For \(^{3}\text{H}-\text{TdR}\) uptake of myeloma cells, there was no significant difference between cells from stage I \((n = 9, 6,246 \pm 2,494 \text{ cpm}; \text{mean} \pm \text{SE})\) and those of stage III \((n = 20, 5,054 \pm 1,338 \text{ cpm}); \(P > .05\)). Furthermore, neither did M-protein secretion rates have any association with clinical stages (stage I \(25.7 \pm 4.9 \text{ pg/cell/day}\), stage III \(24.1 \pm 4.3 \text{ pg/cell/day}\), \(P > .05\)). Thus, proliferation and M-protein secretion activities in myeloma cells have no correlation within each cell and are also variable between cells from different patients.

DISCUSSION

We devised a method for separation of myeloma cells from BM aspirates in MM. The procedure we describe is valuable for isolating myeloma cells of high purity even from BM aspirates containing a low percentage of myeloma cells. Percoll discontinuous gradient centrifugation following Ficoll-Hypaque sedimentation is a useful step in removal of contaminating erythroblasts, monocytes, and myeloid cells. For BM aspirates containing myeloma cells at a very high percentage (>80%), highly purified myeloma cell fractions (>95%) could be obtained only after this Percoll gradient centrifugation (data not shown).

Few reports have described separation of myeloma cells from BM aspirates of patients with MM. Ali and co-workers\(^{10}\) demonstrated that complement-mediated lysis of BM cells from patients with MM using a rabbit antiserum raised against normal peripheral blood mononuclear cells resulted in fractions of highly enriched abnormal plasma cells. They obtained cell suspensions containing 72% to 99% (mean 84.3%) plasma cells with viability which ranged from 65% to 95% following the above-mentioned treatment with antisera plus complement.\(^{10}\) In comparison with their
procedure, the one we describe is multistep; however, it is reproducible and can provide a high purity (>90%) of myeloma cells with good cell viability (>85%) even in BM aspirates containing a low percentage of myeloma cells.

In this study, highly purified myeloma cells were prepared from 29 patients with MG myeloma. Spontaneous proliferation and M-protein secretion in vitro were then investigated. The amount of the secreted M-protein in culture supernatants increased in a time-dependent manner within at least 48 hours of the culture period, and M-protein secretion rate ranged from 4.0 to 72.0 pg/cell/day. Salmon also noted a variation of M-protein synthetic rates from unpurified myeloma cells in vitro (in the presence of other BM cells). Our results suggest that the observed variations in M-protein secretion could result from the innate potential of M-protein synthesis by the myeloma cells when removed from regulatory factors—not only regulatory T cells and monocytes but also cytokines.

Spontaneous proliferation of myeloma cells was evaluated by \(^{3}H\)-TdR incorporation. No significant increase in cell numbers was observed (within ±10%, data not shown) after 48 hours of culture in most of the myeloma cells examined, but the value of spontaneous \(^{3}H\)-TdR uptakes varied significantly (255 to 24,132 cpm/4 \times 10^5 cells) among cells from individual patients. Although \(^{3}H\)-TdR uptake as well as M-protein secretion rate of myeloma cells in vitro had no association with clinical stages (Fig 4), proliferation and M-protein secretion activities were considered independent biologic parameters. Figure 3 shows that there was no correlation between \(^{3}H\)-TdR uptake and M-protein secretion rate in cells of each patient, suggesting that individual myeloma cells possess an endogenous potential to secrete M-proteins without regard to their spontaneous proliferation activity.

The mechanism of growth and M-protein secretion in myeloma cells and its regulation is an important issue which must be investigated. We have studied the effects of a T-cell–derived cytokine, human B cell stimulatory factor 2 (BSF-2/interleukin-6), \(^{18,19}\) which is reported to have growth-promoting activity for murine plasmacytoma and hybridoma cells, \(^{20,21}\) on proliferation and M-protein secretion in freshly isolated myeloma cells, purified as described. Results indicate that BSF-2 can augment proliferation but not M-protein secretion (O. Tanabe et al, manuscript submitted) and that human myeloma cells produce BSF-2 and constitutively express its receptor. \(^{16}\) Therefore, we consider that our method of separating human myeloma cells from patients could contribute significantly to investigation of the mechanism of growth and M-protein secretion in human myeloma cells as an addition to ongoing molecular biologic studies. \(^{22}\)

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Separation of human myeloma cells from bone marrow aspirates in multiple myeloma and their proliferation and M-protein secretion in vitro

K Iwato, M Kawano, H Asaoku, O Tanabe, H Tanaka and A Kuramoto