A Model that Reproduces Syndromes Associated with Human Multiple Myeloma in Nonirradiated SCID Mice (44479)

BEVERLY E. BARTON,*[,1] JENNIFER CULLISON,† JAMES JACKSON,‡ AND THOMAS MURPHY*

Department of Surgery, University of Medicine and Dentistry–New Jersey, Newark, New Jersey 07103; †Department of Allergy and ‡Department of Immunology, Schering-Plough Research Institute, Kenilworth, New Jersey 07033

Abstract. **A human myeloma line was used to create a model of human multiple myeloma *in vivo* that would reproduce the pathophysiology of the disease, including the cachexia associated with cancer. Unirradiated severe combined immunodeficient (SCID) mice were used as surrogate hosts for *in vivo* experiments that allowed the effects of autocrine (human) verus paracrine (murine) cytokines on the development of myeloma to be studied. Serum levels of human paraprotein increased over time and with the number of cells transplanted. Transplanted mice developed major syndromes, cachexia and paralysis (due to invasion of bones by myeloma cells), associated with multiple myeloma. Analyses of serum samples obtained from transplanted mice revealed that when the mice were terminal, total serum protein decreased on average by 20%, whereas serum triglycerides decreased on average by 50%. These data indicate the mice were cachectic, which was confirmed by necropsy. The mice had low but measurable levels of both human and murine interleukin (IL)-6, soluble IL-6 receptor, and murine IL-10 in their sera. The presence of these cytokines and the IL-6 receptor in sera are also characteristics of human myeloma in patients. Since human cells do not respond to murine IL-6, it was possible to demonstrate clearly the importance of autocrine IL-6 in establishing myeloma *in situ*. By reproducing both the hallmarks of a cancer as well as the accompanying paraneoplastic syndromes, this model should be useful in designing more effective therapies for both the primary cancer as well as the accompanying secondary diseases.** [P.S.E.B.M. 2000, Vol 223]

Multiple myeloma is a uniformly fatal B cell neoplasia affecting 9000 people in the United States each year and representing about 1% of all cancer deaths (1). The five-year survival rate has remained at 20%–25% for several years now, despite advances in cancer chemotherapy that have improved survival for other hematopoietic neoplasias.

Myeloma is a cytokine-driven neoplasm, with IL-6 playing a major role in the development of the disease and its manifestations (2–4). Still, the mechanism of IL-6 activity and the effects of IL-6 on both the myeloma cells and the host are controversial. The inability to grow primary myeloma cells *in vitro* limits the ability to study directly the role of IL-6 as well as the potential effects of other cytokines on the tumor cells or the host. Investigations into the biology and therapy of myeloma have also been hindered by lack of an animal model sufficiently like the human disease. BALB/c plasmacytoma lines induced by mineral oil injection (5, 6) have been used, but the resulting tumors do not invade bone. Furthermore, the observation that the development of BALB/c plasmacytomas depends in part upon the cleanliness of the vivarium where the animals are housed indicates distinct dissimilarity to human multiple myeloma (7). To overcome these difficulties, human myeloma cells grown in SCID mice were used to develop a suitable model for studying the biology of myeloma in an *in vivo* environment (8–11). In these studies, successful transplantation depended upon prior irradiation of the mice, a procedure that profoundly distorts the stromal environment myeloma cells

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[1] To whom requests for reprints should be addressed at Department of Surgery MSB G-519, UMDNJ New Jersey Medical School, 185 S. Orange Ave., Newark, NJ 07103. E-mail: bartonbe@umdnj.edu

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require. In some of these models, human paraprotein was not secreted throughout the course of the experiments, which may indicate that the myeloma cells either changed phenotype or were destroyed by the innate immune system, which remains intact in SCID mice (8). Clearly a reproducible model more closely resembling human disease would aid in the development of successful therapy.

By transplanting more myeloma cells per mouse, and waiting longer periods for paraprotein to be detectable in the sera of transplanted mice, we were successful in transplanting human myeloma cells into unirradiated SCID mice. Human paraprotein was measured in the sera. Decreases in serum levels of human paraprotein, total protein, and triglycerides were observed to correlate with cachexia when the disease was advanced. Histologic examinations at these times showed the myeloma cells were still present in the organs of the mice, especially in the bones. Many of the mice became successively paraplegic then quadriplegic, at which point they were euthanized. Thus this model reproduces important symptoms associated with the disease as it occurs in humans.

Materials and Methods

Multiple Myeloma Line. The human multiple myeloma line ARD was used. Its characteristics have been reported previously (12). The line was maintained in complete medium, which consisted of RPMI-1640 (GIBCO, Bethesda, MD) supplemented with fetal calf serum (10% final concentration; Hyclone) treated twice with dextran-coated charcoal (to adsorb serum corticosteroids), 2 m*M* L-glutamine (GIBCO), and 2 m*M* HEPES (GIBCO). Cell-free supernatant fluid from the lines (cultured at 5×10^5 cells/ml) were used to determine if the cells secreted human immunoglobulin and which isotype it was.

Mice. Male CB.17/ICr-*scid* (SCID) mice, 6–8 weeks of age, were obtained from Charles River Laboratories (Wilmington, MA). They were housed and monitored as required in our animal facility. Experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of UMDNJ.

***In Vivo* Transfer of Cells.** Prior to injection in mice, cells were harvested by centrifugation and washed twice with HBSS (GIBCO). The viability of washed cells was determined using the fluorescent viability stain fluorescein diacetate (13), and was determined to be greater than 95% in all experiments. The required number of viable cells was injected into tail veins using 26-gauge needles and 1 ml syringes. Successful transplantation was determined and monitored by measuring the human κ light chain content of the sera weekly, beginning on Day 0. Sera were obtained for these studies by venipuncture through the retroorbital sinuses while the mice were anesthetized with Metofane. Paraproteinemia was defined as serum human κ light chain concentrations of 20 ng/ml or greater. When quadriplegia was observed, or large visible or palpable tumors that interfered with normal functions were observed, the animals were euthanized. In five experiments, success of tumor transplantation was confirmed by histology, necropsy, and gross anatomical examination. In some studies, samples of liver, femur, vertebrae, lung parenchyma, and sternum were collected, preserved in neutral-buffered formalin, routinely processed, and examined by light microscopy. These examinations were performed by board-certified veterinary pathologists.

Determination of Human Kappa Light Chain Levels. The ELISA for the determination of human κ light chain concentration in SCID serum was developed in our laboratory. ELISA reagents were purchased from The Binding Site. Plates were coated with 50 μl per well of Ab (affinity-purified sheep anti-human κ light chain at 4 μg/ml) and allowed to sit at room temperature overnight. After thorough washing with DPBS, 50 μl of sample (appropriately diluted in DPBS/0.5% Tween/0.1% BSA) were added to each well, and the assay was incubated at room temperature for 2 hr. Standard consisted of free human κ light chain, 50 μl/well added at concentrations ranging from 0.3 ng/ml to 300 ng/ml. These were incubated for 2 hr at room temperature as well. After washing with DPBS/Tween, plates were incubated with 50 μl/well of biotinylated Ab (affinity-purified antihuman κ light chain at 0.5 μg/ml) for 1 hr. Fifty μl of streptavidin-horseradish peroxidase were added to each well after washing, and allowed to bind for 1 hr. After a final washing, 50 μl/well of 2, 2′-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) plus hydrogen peroxide (a 1:1 mixture) were added. Absorbance at 405 nm was determined on a VMax ELISA reader (Molecular Devices, Menlo Park, CA). Deltasoft II software was used to resolve data using 4-parameter analysis.

Determination of Serum Levels of Human and Murine IL-6, Soluble IL-6 Receptor, and IL-10. ELISA kits were purchased from Biosource International (Camarillo, CA) and used as per manufacturer's instructions.

Determination of Cachexia During Course of Experiments. Clinical chemistry reagents for the determination of total protein and triglycerides in serum were obtained from Sigma Chemical Company. The protocol for protein determination was modified so that analyses could be performed in microtiter wells, which were read by the plate reader as described above. Standards for total protein and triglycerides were purchased from the supplier, and pretransplant sera were included as controls as well.

Results

Kinetics of Myeloma Lines Transplanted Into SCID Mice. Intravenous injection of as few as 0.5 million ARD cells resulted in positive paraproteinemia by Day 21. In seven out of seven experiments (involving 101 mice), 100% of the mice had significant amounts of human κ chain in their sera. The serum levels were observed to increase over time, and then to decrease when the mice became moribund (due to progressive paralysis or cachexia; Fig. 1). In general, human κ chain did not appear in the sera until

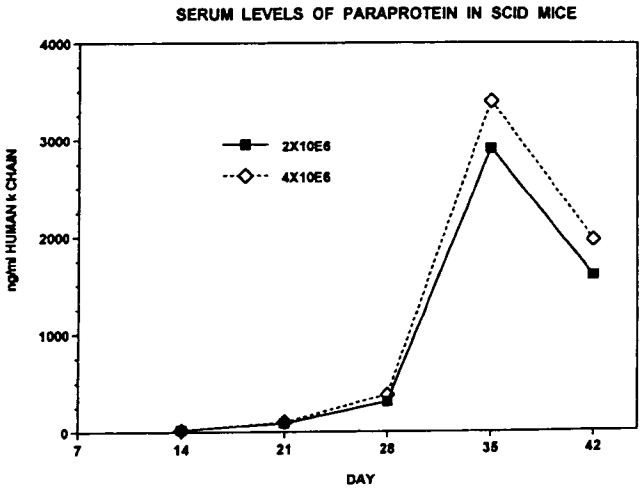

Figure 1. Representative time course is shown of paraproteinemia for human ARD myeloma cells transplanted into SCID mice. Serum samples were obtained weekly and analyzed by ELISA as described in Materials and Methods. Mice were euthanized on Day 42 when all animals appeared moribund, exhibited hind limb paralysis and severe cachexia, as evidenced by the falling paraprotein levels.

about 21 days following transplant depending upon the number of cells injected (Fig. 1). After ≈ 4–6 weeks, mice began to display overt signs of disease, such as hind limb paralysis, visible tumors, cachexia, and palpable masses. Approximately 40% of mice developed hind limb paralysis, whereas about 50% developed visible or palpable tumors. When the mice were obviously moribund, they were euthanized in accordance with our Institutional Animal Care and Use Committee protocol. Thus we did not obtain data relating to potential lethality of the tumor line.

To ensure that the myeloma cells invaded organs, including bones with concomitant displacement of marrow, as seen in human disease, mice were euthanized and necropsied. At this time, several organs were selected for histologic processing and microscopic examination. The presence or absence of the epididymal fat pad was noted. These procedures were performed by veterinary pathologists. The results of the pathology reports are summarized in Table I. All the mice, from five separate experiments, displayed evidence of myeloma cells disseminated in various organs although the cells were transplanted by intravenous injection (Fig. 3). Two mice sacrificed on Day 28 (before overt signs of disease were observed) had myeloma cells only in the liver and lung parenchyma. However, by Day 35, the remaining mice examined had myeloma cells in various organs. Where myeloma cells were observed within marrow cavities, little or no hematopoietic cells were observed. Moreover, samples of vertebrae having myeloma metastases had myeloma cells in the spinal cords and in some of the surrounding striated musculature.

Figure 2 shows the effect of cell number injected on the level of peak paraproteinemia. Injection of 1–4 × 10^6 ARD cells resulted in a peak paraproteinemia in this experiment of 0.2–0.5 μg/ml. Increasing the number of cells to 20 × 10^6 increased the serum κ chain levels to about 2000 μg/ml (Fig. 2). When more than 10 × 10^6 cells were given, paraprotein could be detected as early as 14 days after the injection (data not shown). Based on the data obtained from these early sets of experiments, we used 2 × 10^6 ARD cells as our standard number of cells to transplant for the rest of the experiments described. This number of cells caused enough paraprotein in the sera so that it was detectable by Day 28.

Histology of Transplanted Mice. In five experiments, selected tissues from mice given ARD cells were examined to confirm the successful transplantation of myeloma (Table I). Histopathologic examination following hematoxlin and eosin staining revealed infiltration of anaplastic myeloma-type cells into the marrow cavities of the vertebrae, sternum, and femur of nearly all animals examined, as well as infiltrates into the spinal cords, striated muscle

Table I. Results of Histological Examination of Tissue Obtained at Necropsy

Group ID		Liver	Vertebr.	Sternum	Femur	Lung parenchyma	Epididymal fat pad present
I	A	neg.	pos.	pos.	NA[a]	NA	No
	B	neg.	pos.	pos.	pos.	NA	No
	C	NA	NA	neg.	pos.	NA	No
	D	NA	NA	pos.	pos.	NA	No
II	A	neg.	NA	pos.	pos.	NA	No
	B	NA	NA	NA	pos.	NA	NA
	C	NA	NA	NA	pos.	NA	NA
	D	NA	NA	pos.	pos.	NA	NA
IX	B	pos.	pos.	NA	NA	pos.	NA
XI	A	pos.	pos.	NA	pos.	pos.	No
XIII	A[b]	pos.	neg.	NA	neg.	pos.	Yes
XIII	A[b]	pos.	neg.	NA	NA	NA	Yes

Note. Mice were euthanized at the completion of the experiments; they were necropsied, and their organs were examined histologically by board-certified veterinary pathologists. All work was done blinded. Groups I and II were examined by a different group of verterinary pathologists than groups IX, XI, and XIII. "Positive" indicated the presence of myeloma cells with numerous mitotic bodies.

[a] NA = tissue not examined.

[b] These mice were euthanized at Day 28 instead of after Day 42.

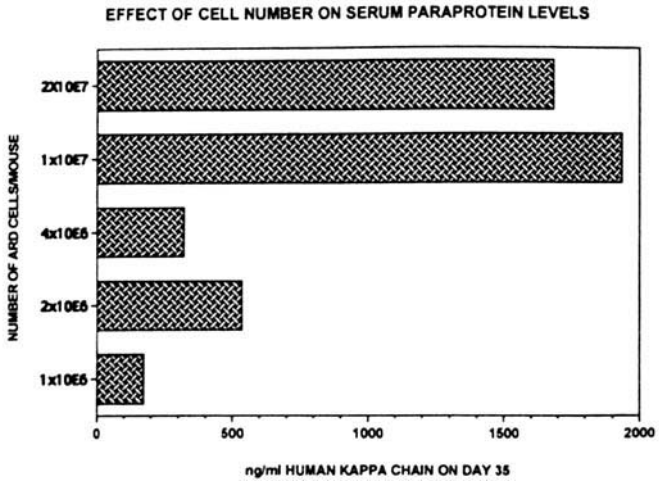

Figure 2. Serum paraprotein levels increased with cell number. Serum paraprotein levels are monitored in patients as a measure of tumor burden. These data demonstrate that the correlation holds true in this model as well. The number of ARD cells indicated were injected on Day 0. Mice were bled once/week. Sera were analyzed for human κ chain, as described. The data obtained on Day 35, before cachexia was apparent, are shown.

surrounding the vertebrae, liver, and lung parenchyma. These were characterized as cells of a highly anaplastic neoplasm with morphologic features of a poorly differentiated malignant myeloma. Two animals that were examined before serum paraprotein was detected had myeloma cells in liver and lung parenchyma, but not in other organs or tissue examined. Neoplastic foci were composed of pleiomorphic mononuclear cells showing marked anaplasia (Fig. 3). Mitotic figures were numerous in all samples examined. The findings are consistent with the human disease.

Cytokines Found in Sera of Transplanted SCID Mice. Table II shows the results of ELISAs performed for both human and mouse IL-6 on the same serum samples. Sera from mice of group VI were pooled. We analyzed these sera for IL-6 of both species because IL-6 could be synthesized either by the tumor cells (human; autocrine) or the stromal cells (murine; paracrine). Low but detectable amounts of IL-6 were found in the pooled samples. The concentrations ranged from 7 to 10 pg/ml for both species. Since the cells are dependent upon autocrine IL-6 for survival, the data for human IL-6 are not surprising. Finding measurable murine IL-6, to which the cells do not respond (data not shown), and which is not found in nontransplanted mice (data not shown), might indicate that myeloma cells serve as a stimulus for stromal cells to secrete IL-6, thereby driving the disease in humans. Low but detectable amounts of murine IL-10 and the soluble murine IL-6 receptor were found as well (data not shown). These findings are similar to what is observed in the human disease.

Development of Cachexia with Time. Table III shows that total protein and total triglycerides in the sera of mice injected with human myeloma cells decreased, which could be interpreted as signs of cachexia. These changes were observed even before decreases in paraprotein were measured (Fig. 1). Both total serum protein and triglycerides were significantly decreased ($P < 0.05$ by Student t test; Table III) at Week 8 compared with the levels measured at Week 2. The decrease in total protein (from 48.3 mg/ml to 39.2 mg/ml; Table III) was observed even while paraprotein in the serum was significantly elevated (19.6 μg/ml, $P <$ 0.005 compared with Week 2; Table III). Necropsy of selected mice showed depletion of fatty stores by Week 8, consistent with the observed decrease in serum triglycerides. The triglyceride levels were observed to rise by 4 weeks following transplant, before falling to about half the starting levels (Fig. 4). These results are consistent with known changes in fat metabolism, whereupon catabolism of fatty acids initally releases triglycerides into serum (6). At later times, serum triglyceride levels decrease as they are used as the organism's energy source (6). Not all mice showed overt signs of disease, such as flaccid tail, hind limb paralysis, or palpable tumors at Week 8. Mice that did not receive tumor cells showed no decreases in serum levels of protein or triglycerides (data not shown). Therefore, measurement of total serum protein and triglycerides should be a useful means to monitor the progression of cancer cachexia as it develops. These findings are consistent with cancer cachexia of humans, in which it has been observed that a tumor mass as small as 500 mg could be accompanied by cachexia (14).

Discussion

The results presented demonstrate that tumor-derived IL-6 is sufficient for myeloma to establish in the absence of elevated serum-IL-6, and that a model of human myeloma cells transplanted into SCID mice may be useful for studying mechanisms and therapies for cancer cachexia.

The use of unirradiated SCID mice as hosts to study a neoplasia of human bone marrow cells has the added advantage of revealing the role of tumor-derived versus host-derived growth factors in the establishment of the disease. It has been shown that IL-6 is an important growth factor for myeloma cells (2–4, 15–17), as it is for other B cells (18, 19). Moreover, it has been shown recently that transgenic mice deficient for IL-6 (IL-6 "knock-out" mice) were resistant to the development of plasma cell tumors (20), whereas their heterozygous littermates developed plasmacytomas at frequencies comparable to those of the background strain (21). However, these experiments could not address whether paracrine or autocrine IL-6 is required for the establishment of plasma cell tumors, as the IL-6 deficiency is in all tissue types of the transgenic mice. Therefore a different system is required to examine these questions. Previously it had been shown that the ARD myeloma line was dependent upon IL-6 to prevent apoptosis ((12); Barton and Jackson, unpublished observations). Therefore this line was successfully transplanted into untreated unirradiated SCID mice, as shown by the increase in human paraprotein in sera over

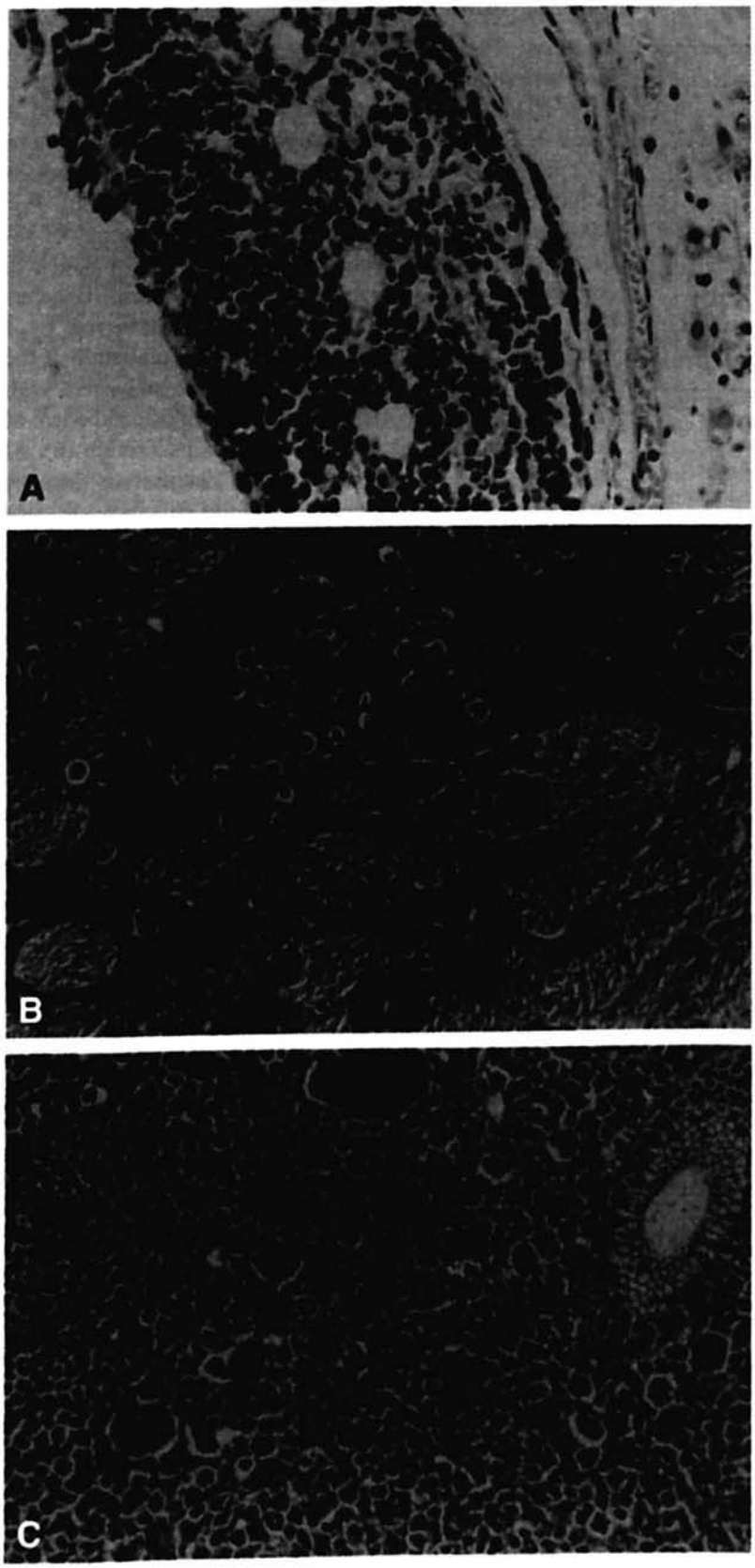

Figure 3. Histology micrographs of various organs obtained at necropsy. (A) Myeloma infiltrate of hind limb (100x); (B) infiltrate of peristernal muscle (125x); (C) infiltrate of epididymis (125x).

time (Fig. 1). Because human cytokines can be distinguished readily from their murine counterparts, we were able to measure both species of the cytokines of interest. It was observed that tumor-bearing SCID mice had measurable amounts of both human and mouse IL-6 in their sera whereas nontumor-bearing mice had no detectable IL-6 of

Table II. Serum Levels of IL-6 in Myeloma-Bearing SCID Mice[a]

Species of IL-6	Cell no.	Serum IL-6 (pg/ml)
Human	4×10^6	10
	2×10^6	10
	1×10^6	10
	none	0
Mouse	4×10^6	10
	2×10^6	7
	1×10^6	7
	none	0

[a] Mice from group VI were bled on Days 0 and 42. To have adequate volume to assay, sera were pooled from each group (10 serum samples per group). Serum IL-6 levels for both mouse and human IL-6 were determined using the Biosource International ELISA kits for murine and human IL-6, following the manufacturer's directions. The lower limit of detection for these kits was 0.3 pg/ml.

Table III. SCID Mice Transplanted with Human Myeloma Cells Develop Clinical Signs of Cachexia Before Paraprotein Levels Decrease

Week	Mean ± SD μg/ml human κ	Mean ± SD mg/ml total protein	Mean ± SD μg/ml total triglycerides
2	0.23 ± 0.29	48.3 ± 1.8	1319 ± 344
8	19.6 ± 5.4[a]	39.2 ± 1.55[a]	521 ± 175[a]
Change	+185%	−19%	−61%

Note. Five SCID mice were given 2×10^6 ARD cells intravenously. Serum was obtained weekly and analyzed by ELISA for human κ chain, and by standard clinical chemistry techniques for total triglycerides and total protein. Week 2 was the time when most of the mice exhibited human κ chain in their sera. Week 8 was the time when the final sera were obtained.

[a] $p < 0.05$ compared with value at week 2 by Student t test.

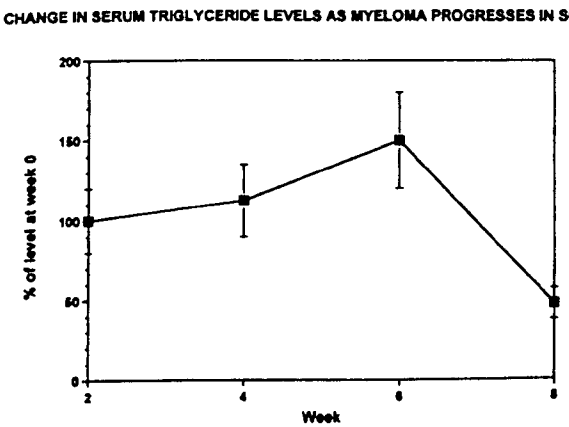

Figure 4. Change in serum triglyceride levels with time in myeloma-transplanted mice. Initially, triglycerides increased with time to approximately 150% of starting levels. As the cachexia progressed, serum triglyceride levels decreased to less than 50% of the starting levels. Values shown are the means ± standard deviations of five mice.

either species in their sera (Table II). These data, presented in Table II, demonstrate that the presence of tumor cells induced host accessory cells to secrete murine IL-6. The serum levels observed in transplanted mice were comparable to those seen in a recent study of myeloma patients (22). Prior to performing the transplant experiments, the cells were analyzed to confirm their dependence on IL-6 and for their ability to synthesize this cytokine. The cells were found to be dependent upon IL-6, which they synthesized (autocrine-dependence; data not shown). Since human cells do not respond to mouse IL-6 (23), and ARD cells are dependent upon the IL-6 they produce for survival (12), it appears that autocrine IL-6 is fully sufficient to sustain the growth and dissemination of the myeloma cells. An autocrine IL-6 loop is known to exist in primary myeloma cells (24–26). Immature myeloma cells were shown to express IL-6 and to produce the cytokine at levels comparable to those produced by the cell lines used in this study (24). The observations presented in this paper indicate that this level of IL-6 production would be sufficient to support the development of the disease. These observations also indicate that elevated IL-6 levels are not a prerequisite for myeloma development, and that the high levels of IL-6 observed in myeloma patients (which reportedly are of prognostic significance (27, 28)), may reflect myeloma-induced increased IL-6 production by accessory cells.

Since in some experiments decreases in serum paraprotein levels were observed (Fig. 1) at the same time necropsy results showed both extensive organ infiltration by myeloma cells and clinical signs of cachexia (absence of epididymal fat pads; Table I), the development of cachexia was monitored by measuring total protein and triglycerides in the sera of animals in advanced disease. Other investigators have used these determinations to monitor cachexia (29). Decreases in total protein and triglyceride levels are among the indications of cachexia (14, 30, 31). Table III and Figure 4 show the results of these studies. Total serum protein levels decreased by 20% on average, even though the paraproteinemia had not yet decreased (Fig. 1 and Table III). Furthermore, total triglycerides in the sera increased when paraprotein first became significant, then decreased to 50% or less of the starting level (Fig. 4). In the hypermetabolic state following rapid glycogen depletion, the fuel source switches from carbohydrates to fats and proteins. The excess fatty acids deposited in adipocytes are catabolized to triglycerides and free fatty acids that circulate in the blood. As the energy requirement increases (during cachexia, for example), the triglycerides are converted to ketone bodies that can be used by the brain directly in place of glucose under these metabolic circumstances (30, 32). The correlation between serum triglyceride levels and body lipid content in cancer cachexia was demonstrated in a mouse lung carcinoma model (33); our serum triglyceride data are comparable to what these investigators observed. It is known that hormonally regulated intracellular lipases are responsible for the catabolism of lipids (30, 32); however, the complete regulation of this enzyme system in cachexia is not understood. The data presented in this paper demon-

strate the utility of this model for experiments designed to understand better how cachexia arises from dysregulation of catabolic enzymes systems. Many other models of cancer cachexia described in the current literature rely upon genetically manipulated tumors in order for cachexia to develop (34–39), which may introduce artifacts not found in cancer cachexia as it occurs in patients.

In summary, these data demonstrate that i) autocrine IL-6 is sufficient for the establishment of multiple myeloma in a host, whereas paracrine IL-6 may serve to amplify the disease process; ii) bone lesions and organ infiltration typical of human myeloma occurred in a mouse model wherein the mice were not irradiated prior to tumor transplantation; and iii) cachexia developed as the cancer progressed. Future experiments are planned to exploit this model for mechanistic studies on the development of cancer cachexia.

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