Erythropoietin induces tumor regression and antitumor immune responses in murine myeloma models

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Recombinant human erythropoietin (rHuEpo) has been used successfully in the treatment of cancer-related anemia. Clinical observations with several patients with multiple-myeloma treated with rHuEpo has shown, in addition to the improved quality of life, a longer survival than expected, considering the poor prognostic features of these patients. Based on these observations, we evaluated the potential biological effects of rHuEpo on the course of tumor progression by using murine myeloma models (MOPC-315-IgAλ_2 and 5T33 MM-IgG$_{2b}$). Here we report that daily treatment of MOPC-315 tumor-bearing mice with rHuEpo for several weeks induced complete tumor regression in 30–60% of mice. All regressors that were rechallenged with tumor cells rejected tumor growth, and this resistance was tumor specific. The Epo-triggered therapeutic effect was shown to be attributed to a T cell-mediated mechanism. Serum Ig analysis indicated a reduction in MOPC-315 λ light chain in regressor mice. Intradermal inoculation of 5T33 MM tumor cells followed by Epo treatment induced tumor regression in 60% of mice. The common clinical manifestation of myeloma bone disease in patients with multiple-myeloma was established in these myeloma models. Epo administration to these tumor-bearing mice markedly prolonged their survival and reduced mortality. Therefore, erythropoietin seems to act as an antitumor therapeutic agent in addition to its red blood cell-stimulating activity.

Erythropoietin (Epo) is a hematopoietic growth factor that is produced in the kidney and regulates red cell production in response to hypoxia (1). As a hormone, Epo circulates in the blood, reaches the bone marrow, and stimulates erythropoiesis by interaction with receptors on the surfaces of erythroid progenitor cells, promoting their proliferation and differentiation and maintaining their viability (1). The cloning of the Epo gene (2) led to the introduction of recombinant human Epo (rHuEpo) into clinical practice in the treatment of several types of anemia, including anemia of end-stage renal disease (3) and cancer-related anemia (4–6).

Multiple myeloma (MM) is characterized by clonal proliferation of bone marrow-transformed plasma cells secreting paraproteins that can be detected in the serum and/or the urine (7, 8). Most patients die of MM or its complications; the median survival ranges from 15 to 48 months (8). About 60–90% of patients with MM develop anemia, the main cause of which is inadequate Epo production (9). rHuEpo has been shown to be effective in the treatment of MM-associated anemia, resulting in a response rate of 70–80% (4, 9–11). In a clinical trial by Mittelman *et al*. (11), five patients received rHuEpo for their anemia for 42–74 months, which was the only therapy for most of the follow-up period. Despite poor prognostic features, these patients survived longer than expected, and their disease has been stabilized. Thus, we hypothesized that, in addition to the correction of anemia, Epo may have an additional hitherto unrecognized biological effect on the disease. To test this hypothesis, we evaluated rHuEpo treatment in two different murine myeloma models.

Murine plasma cell tumors that share some common features with human MM have been used as models for the disease. One such example is a mineral oil-induced plasmacytoma in BALB/c mice—designated MOPC-315 (12)—that has been used for the study of the clinical and immunological aspects of human myeloma (13–15). Similar to the human myeloma cells, the *in vivo* passaged MOPC-315 tumor cells synthesize and secrete a monoclonal Ig (IgA λ_2), thereby providing a measurable marker of tumor load (serum protein component) during tumor progression. MOPC-315 tumor cells correspond to human MM also in relation to morphological and functional heterogeneity of subpopulations, reflecting different stages of differentiation (13). Another unique murine myeloma model—5T MM (IgG$_{2b}$)—developed by Radl *et al*. (16) originated spontaneously in the bone marrow of old C57BL/KaLwRij mice with a frequency of 0.5%. Several tumor lines have been maintained *in vivo* in syngeneic recipients by i.v. transfer of bone marrow. This mouse model resembles the human disease in several aspects: spontaneous origin in the bone marrow, monoclonal gammopathy, and osteolytic bone lesions and destruction. In the present report, we describe the systemic administration of rHuEpo as a potential therapy for MM.

Materials and Methods

Mice. Female mice of the inbred strain BALB/c (The Jackson Laboratory) were used at the age of 6–8 weeks. The C57BL/KaLwRij mice were purchased from Harlan CPB (Zeist, The Netherlands). Male and female mice were 8–10 weeks old when used. All animal procedures were approved by the Weizmann Institute Animal Care Committee.

rHuEpo. The rHuEpo (Epoetin-α or Eprex) used in these studies was obtained from Janssen-Cilag (Baar, Switzerland).

Irradiation and Inoculation. Recipient mice were exposed to 1.7 or 3 Gy of radiation, an exposure of 0.65 Gy/min in a Lucite tube, at a focal distance of 75 cm from a γ-beam 150 A^{60}Co source (Atomic Energy, Canada). Inoculation occurred 2–3 h after total body irradiation by a single i.v. injection of 5×10^2 tumor cells in the tail vein.

Experimental Design to Evaluate Epo-Induced Tumor Regression. The MOPC-315 tumor was maintained by successive i.m. transplant generations in BALB/c mice. Mice were injected s.c. with 10^4 cells in the abdomen area. Epo treatment (administered s.c. at the back flank) was started about 10–13 days after the initial tumor challenge, when a small palpable tumor (2–5 mm diameter) was

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Abbreviations: Epo, erythropoietin; rHuEpo, recombinant human Epo; MM, multiple myeloma; CSF, colony-stimulating factor; DNP, 2,4-dinitrophenyl; SCID, severe combined immunodeficient.

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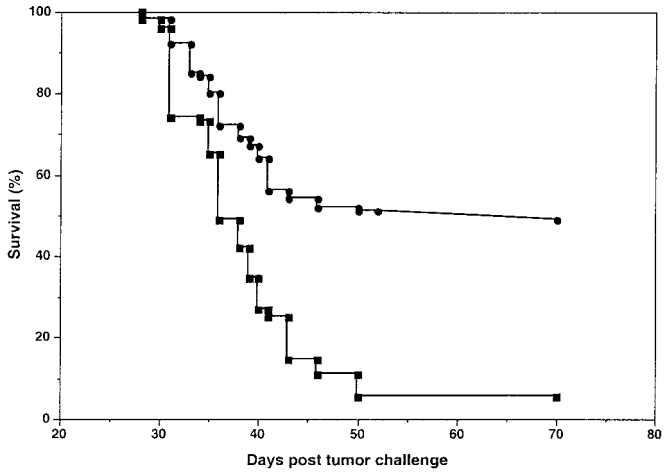

Fig. 1. Induction of tumor regression by Epo. Composite results of five independent experiments, comparing survival of tumor cell-inoculated mice treated with Epo (begun 11–13 days after tumor-cell challenge) with survival of control mice treated with the diluent. Mean complete tumor regression was observed in 31 of 61 mice (51%) vs. 3 of 55 (5.4%) in the controls ($P < 0.05$, Kolmogorov–Smirnov test, one tail). Square, MOPC-315 + diluent; circle, MOPC-315 + Epo.

observed. Control groups were injected with the diluent (sterile water for injection from B. Braun, Melsungen, Germany). The tumor growth rate of individual mice was monitored twice weekly during Epo or diluent administration. To establish optimal treatment modalities affecting tumor development, we tested the effects of different regimens with various rHuEpo doses (10, 20, 30, 40, 50, and 100 units per injection) and time schedules (daily treatment for 10 or 20 consecutive days, or three injections per week for 4 weeks). Maximal tumor regression was obtained after daily s.c. injections of 30 units of Epo for 10 consecutive days, followed by three times per week for an additional 2–3 weeks. This Epo-treatment schedule was used in all of the experiments described in this study.

Western Blot Analysis. The analysis was performed essentially as described by Neumann *et al.* (17). Polyclonal rabbit antibodies directed against the murine λ light chain (from ICN) were used at 1:1,000 dilution for immunoblotting. Donkey anti-rabbit Ig conjugated to horseradish peroxidase was obtained from Amersham Pharmacia.

Measurement of Hb Levels. Blood samples were drawn into Vacvette (Greiner Labortechnik GmbH, Kremsmunster, Germany) test tubes containing EDTA-k_3. The Hb level was determined by the H_2-Technicon Coulter (Dublin, Ireland).

Results

Epo-Induced Tumor Regression. Mice challenged with a syngeneic progressive growing tumor (10^4 cells administered s.c.) were treated with rHuEpo by systemic administration. Tumor growth rate over the period of 14–18 days after tumor-cell challenge was similar in all mice, but there was a decrease thereafter in tumor size in mice responding to Epo, resulting in a permanent and complete tumor regression in 30–60% of the Epo-treated mice (observed in a total of 150 mice; Fig. 1). Once tumor regression was established, no more Epo treatment was required for the maintenance of regression. Tumor recurrence was observed in only 3 of 20 regressors kept for a follow-up period of 12 months. Spontaneous tumor regression in the control groups injected with the diluent was between 0–10% (observed in a total of 100 mice). Tumor progression in mice not responsive to Epo treatment, culminating in their deaths, was observed shortly after termination of Epo treatment (30–50 days after tumor cell challenge); no significant effect on the progressive tumor growth rate was observed. Thus, "regressor" refers to a mouse injected with tumor cells, whose condition was characterized first by initial tumor growth and then gross disappearance of the tumor cells after Epo treatment. "Progressor" refers to a mouse injected with tumor cells, whose condition was characterized by continuous tumor growth irrespective of Epo treatment.

In one experiment, we compared the effect of rHuEpo treatment on tumor growth vs. administration of another hematopoietic growth factor, rHuG-CSF (recombinant human granulocyte colony-stimulating factor). Because Epo is a large glycosylated molecule, we used Lenograstim, a glycosylated G-CSF (Granocyte, Chugai Pharma). The treatment schedule (involving the same protein levels) was the same as for Epo administration (250 ng, equivalent to 30 units of Epo); the injected dose of rHuG-CSF was 260 ng per injection. This growth factor failed to produce tumor regression; rather, it enhanced tumor progression and also increased tumor size (data not shown). In contrast, a 50% tumor regression in Epo-treated mice was observed in this experiment.

The possibility that Epo exerts its therapeutic effect through binding to cell-surface receptors on the MOPC-315 cells was examined (17). However, no specific binding of ^{125}I-Epo was observed (data not shown), thus suggesting that the effect of Epo on tumor regression involves an indirect mechanism.

Hb Levels Affected by Epo Treatment of BALB/c Mice. In humans, the erythroid response to Epo is characterized by an increase in Hb levels. Hb levels were tested in groups of normal BALB/c mice or tumor-bearing mice (8–10 mice per group), with or without Epo treatment. Measurement was done 30 days after the start of Epo administration. Epo administration increased the Hb levels in normal BALB/c mice from a mean of 13.6 g/dl (range 12.7–15.3) to a mean of 20.9 g/dl (range 19.8–21.7). Among regressors treated with Epo, seven of nine mice showed elevated levels ranging from 17 to 21.5 g/dl (mean 18.4), and two of nine had lower levels (close to normal values of 13.8 and 14.5). Hb levels in two spontaneous regressors were 16.2 and 16.4 g/dl. In 9 of 11 progressor mice treated with Epo, Hb levels ranged from 11.5 to 13.4 g/dl (mean = 12.6), whereas in 2 of 11 progressors that carried a small tumor load, the levels were 16.3 and 16.7 g/dl. In two progressors treated only with diluent, the Hb levels were 12.5 and 13.4 g/dl. Thus, Epo increases Hb levels in normal and regressor mice.

Elevated λ Light Chain in Sera of MOPC-315-Injected Mice. The therapeutic effect of Epo on tumor progression prompted us to compare the levels of MOPC-315-secreted light chain in sera of progressor and regressor mice as markers for the state of the tumor. Immunoblot analysis with rabbit anti-mouse λ light chain antibodies displayed a specific doublet (two closely migrating bands) at ≈22 kDa in sera from MOPC–315 injected mice (Fig. 2). The specificity of this doublet as a λ light chain was confirmed by the lack of reactivity of sera from MPC-11 (IgG_2a)-injected mice (18) and from a normal age-matched control mouse with the rabbit anti-mouse λ light chain antibodies (Fig. 2*A*, lanes 12 and 13, respectively).

The faster migrating band of the 22-kDa doublet (Fig. 2, open arrow) was more prominent in sera from progressor mice (Fig. 2*A*, lanes 2–4) and comigrated with the λ light chain of purified Ig derived from MOPC-315 cells (Fig. 2*A*, lane 1). The third lower molecular mass band (observed in Fig. 2*A*, lanes 2–4) was also observed in the MOPC-315-secreted Ig on longer exposure periods (data not shown) and may represent a proteolytic fragment of the λ light chain. The slower migrating 22-kDa band (solid arrow) was more prominent than the faster migrating band (open arrow) in sera from regressor mice 2 months after the tumor injection (Fig. 2*A*, lanes 5–11), and was also apparent in sera from control mice (14 months; Fig. 2*B*, lane 7). The profile of the λ light chain in the regressor mice was maintained irrespective of the duration of regression (2–15 months; Fig. 2*B*).

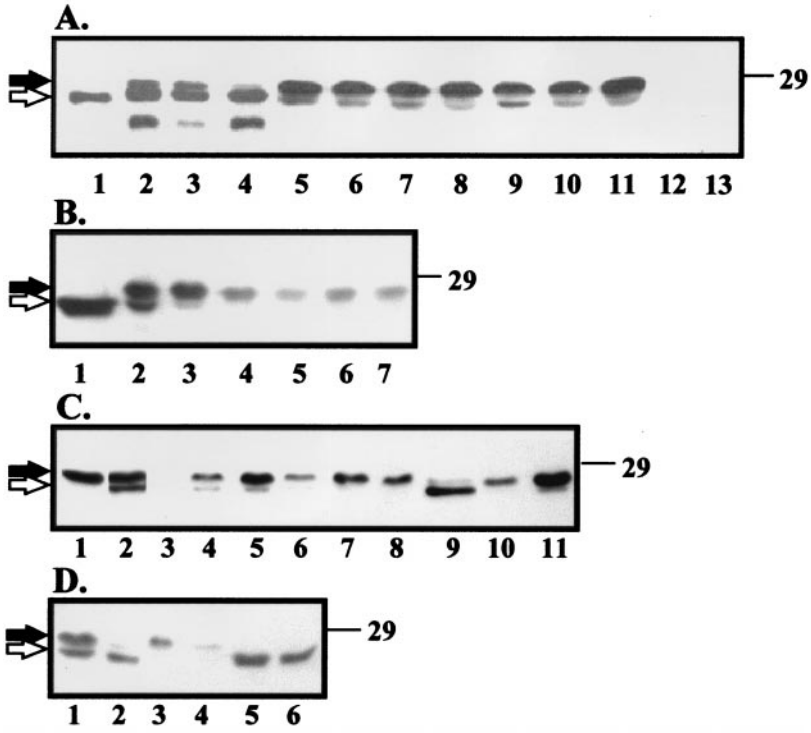

Fig. 2. Western blot analysis of sera from tumor-injected mice. (*A*) Western blot analysis of serum proteins from MPC-11- and MOPC-315-injected mice. Sera (2 μl diluted 1:1 in 0.9% NaCl) from progressor mice injected with MOPC-315 (lanes 2–4), a spontaneous regressor mouse (lane 5), regressor mice treated with EPO (lanes 6–11), MPC11-injected mouse (lane 12), and a control (nontreated) mouse (lane 13), as well as purified Ig from MOPC-315 (lane 1) were resolved by SDS/10% PAGE, and immunoblotted with rabbit anti-mouse λ light chain antibodies (ICN). The enhanced chemiluminescence (ECL) method was used for detection essentially as described (19). The 29-kDa molecular mass marker is depicted on the right. (*B*) Profile of λ light chain in regressor mice, with respect to time of regression. Regressor mice were bled 2, 6, 12, and 15 months after MOPC-315 cell injection (lanes 3–6, respectively). Serum from an old (12 months) age-matched control (lane 7), purified Ig from MOPC-315 cells (lane 1), and serum from a progressor (lane 2) are depicted for comparison. Sera were resolved by SDS/10% PAGE and analyzed by Western blotting with rabbit anti-mouse λ light chain antibodies, by using ECL for detection. (*C*) Profile of λ light chain with respect to the viability of injected tumor cells and the time after injection. Representatives of three groups of five mice each on which the experiment was performed are depicted. Mice were bled before tumor-cell injection (lane 3) and 14 days after injection with 10^4 live cells (lanes 4 and 6), with 10^6 cells irradiated with 10 Gy (lane 8), or with 0.3 ml of ascitic fluid from an MOPC-315-bearing mouse (lane 10). Mice were bled a third time, 14 days after the second bleed (lanes 5 and 7; lane 9 and lane 11, respectively). Sera from regressor (lane 1) and progressor (lane 2) mice are depicted for comparison. Western blot analysis was performed as described. (*D*) Binding of sera from mice to 2,4-dinitrophenyl (DNP)-Sepharose. Western blot analysis with rabbit anti-mouse λ light chain antibodies. Sera from mice (5 μl) were incubated with 20 μl of DNP-Sepharose (1:1 slurry) for 1 h at 4°C. The beads were washed three times in PBS containing 0.5% Triton X-100, 0.5% deoxycholate, and 0.1% SDS, followed by two washes in PBS. Samples were then eluted with Laemmli sample buffer and separated on SDS/10% PAGE, followed by Western blot analysis. Lanes 1, 3, and 5 represent sera from progressor and regressor mice as well as MOPC-315 Ig before absorption on DNP-Sepharose. Lanes 2, 4, and 6 represent the eluted samples, respectively.

Tumor Progression Correlates with Elevated MOPC-315 λ Light Chain in the Serum. The correlation between tumor progression and the presence of the faster-migrating λ light chain is further demonstrated in Fig. 2*C*. Mice were injected with 10^4 MOPC-315 cells or with 10^6 MOPC-315 cells irradiated with either 10 Gy or ascitic fluid from a MOPC-315 tumor-bearing mouse. Mice injected with irradiated cells or with ascitic fluid did not develop tumors and displayed only the slower migrating λ light chain band (Fig. 2*C*, lanes 8 and 10). At 14 days after injection, these mice were challenged with 10^4 MOPC-315 cells, and they all developed either local or disseminated tumors. In these tumor-bearing mice, the faster migrating λ light chain band was evident (Fig. 2*C*, lanes 9 and 11). Mice injected with the viable MOPC-315 cells developed a palpable tumor (Fig. 2*C*, lane 4) and became progressors (Fig. 2*C*, lane 5). One of the mice injected with viable cells developed a smaller tumor (Fig. 2*C*, lane 6) and eventually became a spontaneous regressor (Fig. 2*C*, lane 7). Note the presence of the faster migrating band in lanes 4 and 6 and its absence in lane 7; a faint, faster migrating band could be detected in lane 6 on longer exposure periods (data not shown). Hence, the lower 22-kDa band observed in progressor sera represents the λ light chain of the IgA secreted by the MOPC-315 cells. Its high intensity reflects expansion of the tumor mass in progressor mice; the lower intensity of this band in sera from regressor mice correlates with gross tumor disappearance. The slower migrating 22-kDa band, evident in sera from regressors, most probably reflects an immune response against the MOPC-315 cells, which precedes the appearance of the faster 22-kDa migrating band. The slower migrating 22-kDa band was occasionally observed in the age-matched 12-month-old control mice (Fig. 2); the 22-kDa band probably represents the endogenous-Ig light chain.

Because MOPC-315 cells secrete DNP-specific antibodies, we tested further the antigenic specificity of the λ light chain in sera of regressors and progressors by employing DNP-lysyl Sepharose (DNP-Sepharose). As can be seen in Fig. 2*D*, the lower λ light-chain band in sera from a progressor mouse (lane 1) specifically bound to the Sepharose (lane 2) and comigrated with the λ light chain of MOPC-315 (lane 5), which also bound specifically to the DNP-Sepharose (lane 6). The slower migrating band at 22 kDa in the sera of a regressor mouse (lane 3) barely bound to the DNP-Sepharose (lane 4). The serological differences between progressors and regressors (as depicted in the profile of the λ light chain) provide a reliable biochemical method to distinguish between progressors and regressors.

Specific Tumor Immune Responses Induced in Epo-Triggered Regressors. Mice in regression for 4, 7, and 15 months were rechallenged s.c. with 10^4 MOPC-315 tumor cells. They rejected this second tumor-cell challenge (a composite of four experiments involving a total of 37 mice) in contrast to normal control mice that developed 100% tumor takes within 30 days. The specificity of this resistance to the growth of MOPC-315 tumor cells was demonstrated further. Regressor mice (4 months in regression) were challenged bilaterally, on the left ventral surface with 10^4 MOPC-315 cells and on the right ventral surface with MPC-11, a different plasmacytoma cell line (also induced in BALB/c mice). Both tumors grew progressively in control mice (all mice died within 30–40 days). In contrast, the MOPC-315 tumor was rejected in 12 of 12 regressor mice, while MPC-11 tumor cells grew progressively on the right-hand side of these same hosts.

T Cell-Mediated Therapeutic Effect of Epo. The potential involvement of the immune system in Epo-triggered tumor regression was tested further by comparing the response to Epo in CD1 nude mice (lacking normal T cell function) and in CB17-SCID (severe combined immunodeficient) mice (severely deficient in functional B and T lymphocytes) challenged s.c. with 10^4 MOPC-315 tumor cells. Mice were treated with Epo or diluent, starting 10 days after

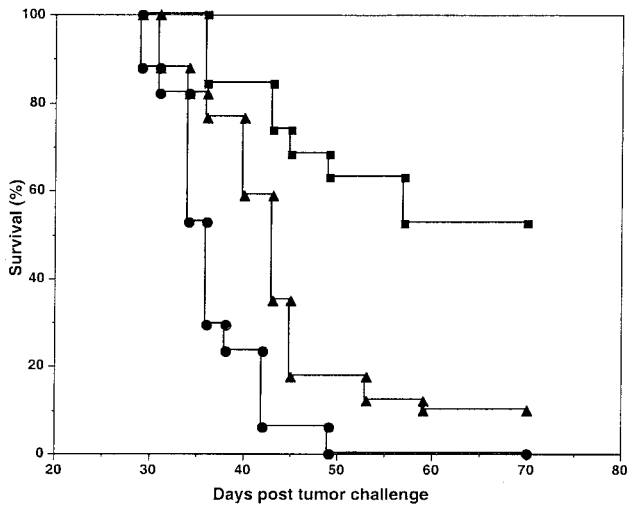

Fig. 3. Phenotype of the effector cells. Normal BALB/c mice or mice depleted of $CD4^+$ or $CD8^+$ cells (after two i.v. injections of mAb within 10 days) were injected s.c. with 10^4 MOPC-315 cells. After tumor-cell challenge (11–13 days), mice were treated with Epo. Tumor regression was observed in 50% of mice in the Epo-treated control group. In contrast, 90–100% tumor growth was observed in Epo-treated mice depleted of $CD4^+$ or $CD8^+$ cells. Square, normal + Epo; circle, anti-CD8 mAb + Epo; triangle, anti-CD4 mAb + Epo.

tumor-cell challenge in SCID mice and 17 days in nude mice. Epo treatment in SCID and nude tumor-bearing mice failed completely to confer tumor regression. All mice (100%) died within 26 days and 37 days, respectively, after the tumor-cell challenge. After Epo administration to SCID and nude mice, Hb levels were elevated similarly to those seen in Epo-treated BALB/c mice.

The Epo-triggered therapeutic effect attributed to a T cell-mediated mechanism was demonstrated further by adoptive transfer of immunity. Different spleen-cell populations from Epo-triggered regressors or from normal mice were separated on nylon–wool columns to adherent (containing B cells and macrophages) or nonadherent (up to 95% T cells) cells. The separated cells (15×10^6) were injected i.v. into the irradiated (5 Gy) recipients; the tumor challenge (10^4 MOPC-315 cells) was performed 24 h after the adoptive transfer of lymphoid cells. Tumor growth was followed for 22 days. Nonadherent cells (up to 95% T cells) from regressor spleens (taken 20 days after establishment of tumor regression) mediated tumor rejection in seven of eight mice (87.5%) in contrast to zero of seven mice receiving adherent cells (containing B cells and macrophages). Nonadherent and adherent spleen cells from normal mice failed to mediate tumor rejection in zero of five and zero of four mice, respectively.

Phenotype of Tumor-Specific Effector Cells. To elucidate the involvement of T cells in the therapeutic effect of Epo further, we tested whether *in vivo* depletion of either $CD4^+$ or $CD8^+$ T cells of MOPC-315 recipients would negate the ability of tumor-bearing mice to respond to Epo treatment. BALB/c naive mice were injected i.v. twice (10 days between injections) with mAb to either CD4 or CD8 T cells, causing depletion of these cells (19). The adequacy of the *in vivo* anti-CD4 and -CD8 antibody treatment was reflected in the levels of $CD4^+$ and $CD8^+$ T cell populations in the spleens of the treated mice. In mice injected with anti-CD4 mAb, only 2% $CD4^+$ cells were present in comparison to 22–28% in spleens of untreated mice. In anti-CD8 mAb treated mice, 2% $CD8^+$ cells were observed vs. 21% in controls. These pretreated mice and control mice were challenged with 10^4 tumor cells s.c.; after initial tumor-cell growth (11–13 days after tumor-cell inoculation), the mice were treated further with Epo. The results are summarized in Fig. 3. Eradication of $CD8^+$ cells abolished the response to Epo treatment; tumor growth was enhanced, and no tumor regression was observed. Elimination of $CD4^+$ cells also negated the curative effect of Epo treatment, although tumor progression was delayed (Fig. 3). Epo treatment caused tumor regression in 50% of the naive BALB/c mice challenged with tumor cells and thereafter receiving Epo injections.

Table 1. The phenotype of the cells mediating tumor elimination

Type of injected cells*	Mice developing tumors	
	Exp. 1	Exp. 2
T alone	4/4–100%†	6/6–100%
T + Norm Ad	4/4–100%	8/8–100%
T + Norm non-Ad	4/4–100%	8/8–100%
T + Reg Ad	4/4–100%	7/7–100%
T + Reg non-Ad	0/4–0%	1/6–17%
T + Reg non-Ad + $CD8^+$ C′	n.d.	4/6–67%
T + Reg non-Ad + $CD4^+$ C′	n.d.	1/6–17%

*MOPC-315 tumor cells (T; $n = 10^4$) were mixed with spleen lymphoid cells at a ratio of 1:250 and immediately injected s.c. (0.1 ml) into BALB/c mice. Spleen cells from regressors (Reg) and normal (Norm) age-matched controls were separated on nylon–wool columns to adherent (Ad) and nonadherent (non-Ad) subpopulations. Part of the Reg non-Ad purified T cells were treated further with either anti-CD4 or anti-CD8 mAbs plus rabbit complement (C′). Spleen cells were taken from Epo-induced regressors 2.5 months in regression (Exp. 1) or 5.5 months in regression (Exp. 2).
†Number of mice with tumors 25 days after MOPC-315 transplantation.

The cell-surface phenotype of the cells mediating tumor elimination was tested further by using the Winn test assay. Different spleen-cell populations from regressors vs. normal mice were mixed with 10^4 MOPC-315 tumor cells and injected s.c. into naive recipients. Nylon–wool nonadherent purified T cells from MOPC-315 regressor spleens (taken from mice 2.5 or 5.5 months in regression) prevented the growth of the MOPC-315 tumor cells. In contrast, adherent cells from regressor spleens and adherent and nonadherent cells from normal spleens failed to prevent tumor growth. Depletion of $CD8^+$ cells of the nonadherent spleen-cell population from regressors with anti-$CD8^+$ mAb plus complement negated the ability of these cells to mediate tumor rejection in 67% of mice. In contrast, transfer of $CD4^+$ T cell-depleted nonadherent spleen cells resulted in an almost complete rejection—only 17% of mice developed tumors (see Table 1).

Development of Myeloma Bone Disease. One serious debilitating complication in about 80% of patients suffering from MM (besides the malignant proliferation of plasma cells in bone marrow) is bone destruction caused by increased osteoclast activity, which results in pain and fractures, as well as decreased bone formation in the areas of bone adjacent to myeloma cells (20). Several investigators have shown that bone disease can be induced in mice when they are exposed to low doses of whole-body irradiation followed by i.v. injection of myeloma tumor cells. Myeloma tumor cells in the irradiated recipients are engrafted in the bone marrow, inducing osteolytic lesions and hind-limb paralysis (as a result of compression of the spiral cord by tumor cells), followed by dissemination to other sites including spleen and liver (21, 22). By using this induction procedure, we obtained bone involvement after i.v. injection of 5×10^2 MOPC-315 i.v. cells into BALB/c mice exposed to 1.7 Gy or 3 Gy whole-body irradiation (within 2 h after irradiation). This treatment caused myeloma bone disease and mortality in 100% of injected mice 28–35 days after tumor-cell injection. Light microscopy indicated tumor-cell infiltration among bone-marrow cells (mimicking the human disease), lesions in vertebrae, long bones with loss of trabeculae, and increased osteoclast numbers. Experiments were performed to test the effect of Epo on this bone disease. Epo treatment was initiated 24 h or 5 days after tumor-cell injection (30 units of Epo for 10 consecutive days followed by three injections per week for an additional 2–3 weeks). In the first set of

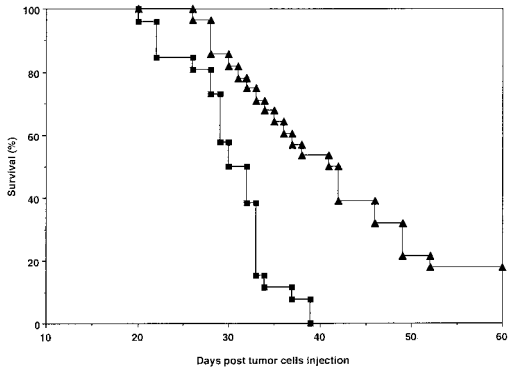

Fig. 4. Myeloma bone disease. Female BALB/c mice exposed to 3 Gy whole-body irradiation were injected i.v. with 5×10^2 MOPC-315. After tumor-cell injections (24 h), mice were treated with Epo (solid triangle) or diluent (solid square). The Epo significantly prolonged the survival of treated mice ($P = 0.0021$, Mann–Whitney *U* test).

experiments, mice were exposed to 3 Gy of irradiation, and Epo treatment was started 24 h after exposure to radiation. A summary of three experiments is shown in Fig. 4. In the control group, 100% mortality (26 of 26) was observed, and 82% (23 of 28) mortality was observed in the Epo-treated mice. Epo treatment prolonged the survival of the sick mice significantly ($P = 0.0021$). In the second set of experiments, mice were exposed to 1.7 Gy of irradiation, and Epo administration was initiated 5 days after exposure to radiation. Bone disease developed in 100% of mice (18 of 18) injected with the diluent and in 85% (17 of 20) after treatment. A significant prolongation of survival ($P = 0.0167$) was observed in Epo-treated mice.

The 5T Myeloma Model. Early-stage human myeloma is primarily a bone-marrow disease, which in late stages metastasizes to a variety of organs. The unique 5T myelomas that Radl *et al*. (16) have described represent a murine plasma-cell disease that occurs spontaneously in the bone marrow of old C57BL/KaLwRij mice. Several transplantable 5T MM cell lines were developed. To study the effect of Epo on tumor progression, we used the *in vivo* propagated 5T33 MM tumor, which is remarkably similar to that found in the human disease.

We tested the effect of Epo after the intradermal inoculation of

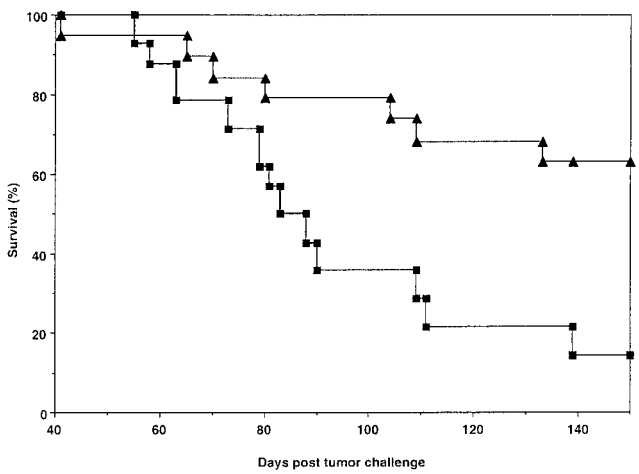

Fig. 5. Tumor regression in the 5T myeloma model. A quantity (10^6) of 5T33 MM cells was injected intradermally into C57BL/KaLwRij mice. When palpable tumors were observed (19 days later), the mice were treated either with diluent (solid squares) or Epo (solid triangles).

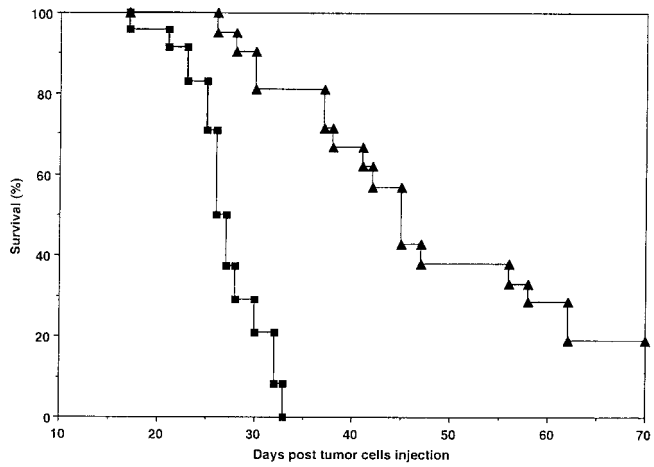

Fig. 6. Bone disease in the 5T33 MM model. C57BL/KaLwRij mice were injected i.v. with 5×10^4 5T33 MM cells. The mice were treated with diluent (solid squares) or Epo (solid triangle) 7 days later. Epo treatment significantly prolonged the survival of treated mice ($P < 0.0001$, Mann–Whitney *U* test).

10^6 5T33 MM cells. Initial tumor growth was observed 19 days after tumor-cell challenge. These mice were divided into two main groups: the control group was injected with the diluent, and mice in the second group received daily injections of Epo (30 units per injection) for 10 consecutive days followed by three injections per week for an additional 7 weeks. We observed 85% tumors in the control group (12 of 14) in contrast to 36% (7 of 19) tumor development in Epo-treated mice (Fig. 5). The Epo-treated regressors were rechallenged with 10^6 5T33 MM tumor cells s.c. on day 135 after the original tumor-cell challenge. We observed tumor-cell takes in 1 of 12 of these injected mice (versus 5 of 5 in normal control mice with a 35-day mean latency).

The effect of Epo on the myeloma bone disease was also tested. Because the tumor cells home to the bone marrow we could avoid the exposure of the recipient mice to radiation. Mice were injected with 5×10^4 5T33 MM tumor cells i.v.; 7 days thereafter, they were given further treatment with 10 daily s.c. injections of Epo (30 units per injection) or diluent followed by 3 injections per week for an additional 2 weeks. The results (involving a summary of four experimental groups) are summarized in Fig. 6. In the control group, 100% mortality was observed (26 of 26); in the Epo-treated groups, the mortality was 82% (17 of 21). Epo treatment significantly prolonged the survival of the tumor-bearing mice ($P < 0.0001$).

Discussion

The main findings of the present research (using the MOPC-315 murine myeloma model) are that Epo, besides its known activity on the erythroid lineage, induces tumor regression by promoting an effective antitumor immune response. Mice challenged with a progressively growing myeloma and treated with Epo for a relatively limited period exhibited complete tumor regression in 30–60% of the cases (Fig. 1). Once tumor regression was established, no more Epo treatment was required for the maintenance of regression. Western blot analysis provided a reliable biochemical method to distinguish between progressor and regressor mice. In the progressor mice, the predominant λ light chain was derived from MOPC-315, because it comigrated with the MOPC-315-specific λ light chain and specifically bound to DNP-Sepharose. In regressor mice, however, the predominant λ light chain migrated in a manner similar to the endogenous mouse λ light chain, reflecting the reduction in tumor mass. It seems that the injection of tumor cells *per se* elicits an immune response in the mice, as judged by the early appearance of λ light chain in MOPC-315-injected mice,

before detection of the MOPC-315 λ light chain. The ability to distinguish between the endogenous mouse λ light chain and the λ light chain secreted by the tumor cells may provide a diagnostic means to predict and follow the progressive states of the tumor. It has been shown that secreted Ig chains can differ in their electrophoretic mobility, depending on their state of glycosylation (23). The M protein in patients with multiple myeloma is a landmark of the disease, and it persists irrespective of the physical state of the patient (24). A future issue to be addressed is whether there are molecular changes in the M protein in the course of the disease with respect to the clinical status of the patient.

Studies of the biological mechanisms involved in Epo-triggered MOPC-315-tumor regression suggest that antimyeloma immunological reactivity is involved in the induction and maintenance of tumor regression. In the regressors, memory of the tumor antigen seemed to be established, because mice 4, 7, and 15 months in regression rejected a second MOPC-315 tumor-cell challenge. This immunologically triggered resistance was shown to be tumor specific. The involvement of T cells in Epo-triggered tumor rejection was indicated by comparing the responses of normal, SCID, or nude mice to Epo treatment after the tumor-cell challenge. Immunologically impaired SCID and nude mice were found to be unresponsive to Epo treatment. Negation of the antitumor response induced by Epo treatment in these T cell-deficient mice rules out the possibility that Epo has a direct cytotoxic or cytostatic effect on tumor cells. Indeed, ^{125}I-Epo binding failed to detect receptors for Epo on the surfaces of the MOPC-315 cells, suggesting that the investigated biological effect of Epo is indirect. The role of T cells in the Epo-triggered tumor regression was demonstrated further by the adoptive transfer of immunity, preventing tumor-cell growth in irradiated mice only after transfer of T cells from spleens of regressors into the irradiated recipients. *In vivo* depletion of $CD4^+$ or $CD8^+$ T cells also annulled the curative effect of Epo treatment, although eradication of $CD8^+$ cells actually enhanced tumor progression. Depletion of $CD4^+$ cells prolonged the median survival time of the Epo-treated tumor-bearing mice (Fig. 3). Further tests to elucidate the cell-surface phenotype of the cells mediating tumor regression suggest that Epo-induced regression promotes the generation of $CD8^+$ effector cells that play a major role in the tumor rejection process (Table 1). However, the *in vivo* elimination of $CD4^+/CD8^+$ cells in tumor cell recipients treated with Epo (Fig. 3) suggests that both $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells contribute to the Epo-dependent tumor regression. $CD4^+$ T cells may act as helpers, releasing helper factors that augment the primed cytotoxic T lymphocyte killing activity of the effector phase (25).

The therapeutic effect of Epo administration on tumor regression was confirmed when the unique 5T33 MM murine myeloma model was tested. The intradermal route of tumor-cell challenge prolonged the latency of tumor development, thus prolonging the Epo treatment for several weeks. Tumor regression was observed in about 60% of the Epo-treated mice (Fig. 5); these regressors rejected the growth of a second tumor-cell challenge, thereby indicating memory of the tumor antigen, similar to our observations with the MOPC-315 studies.

Our experimental myeloma model MOPC-315, using the s.c., i.p., or i.v. routes of tumor-cell injection into normal BALB/c recipients, did not involve infiltration of tumor cells into bone marrow or bone tissue. This problem was overcome by exposing the tumor-cell recipients to a low dose (1.7 or 3 Gy) of total body irradiation; recipients were inoculated further i.v. with 5×10^2 MOPC-315 tumor cells, yielding hind-leg paralysis and mortality in 100% of injected mice within 4–5 weeks. Histologic examination indicated tumor-cell infiltration among bone-marrow cells, bones, and other organs. Host irradiation was a prerequisite for tumor-cell invasion into bones; similar and higher doses (up to 10^6 cells i.v.) of tumor cells injected into normal recipients did not result in bone disease. Several years ago, we showed (26) that host irradiation (similar low doses) triggered the production of cytokines, including IL-6 and CSF-1. IL-6 has been shown to be a myeloma growth factor *in vivo* (27) and is known to stimulate osteoclastic bone resorption and osteoclast formation (28). CSF-1 has been shown to be involved in bone morphogenesis in the mouse (29). These factors—probably secreted by bone marrow stroma—could thus be important for tumor-cell proliferation and infiltration into bone tissue.

The present studies used both murine myeloma models, and the induction of bone disease described herein enabled us to test the therapeutic effect of Epo treatment in these mice. Epo administration (using the treatment schedule described for tumor regression after s.c. tumor inoculation) prevented the development of bone disease in about 20% of the treated mice (vs. 100% mortality in the control group) and significantly prolonged the survival of the tumor-bearing mice.

The present data from our studies with the MOPC-315 model provide evidence that Epo treatment promotes the development of an effective tumor-specific immune response to a mouse myeloma model which is mediated by T cells. Epo may play an immunomodulatory role and be useful as a therapeutic agent. How Epo mediates its effects has not yet been elucidated fully. Recently, it has been reported that systemic administration of rHuEpo functions as a neuroprotective agent in animal models (30). Thus, Epo possesses biological activities in addition to its established erythropoietic effects and seems to have a pleiotropic nature.

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