Brief Definitive Report

INTERLEUKIN 3 PERFUSION PREVENTS DEATH DUE TO ACUTE ANEMIA INDUCED BY MONOCLONAL ANTIERYTHROCYTE AUTOANTIBODY

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IL-3 is known to promote the growth and differentiation of hemopoietic progenitors of the erythroid and myeloid lineages (1, 2). Availability of the recombinant purified protein has led to an examination of its potential as a therapeutic agent for hematological disorders. Since we have recently developed a model of an acute, lethal form of anemia caused by a single injection of a monoclonal anti-mouse RBC (anti-MRBC) autoantibody derived from autoimmune NZB mice (3), we evaluated the protective effect of murine rIL-3; this effect was compared with that of recombinant erythropoietin (rEpo) and granulocyte-macrophage CSF (rGM-CSF). Our results indicate that perfusion of murine rIL-3 completely prevents death due to acute anemia induced by the anti-MRBC mAb. This beneficial effect contrasted with the partial or absent effect of rEpo and rGM-CSF perfusion.

Materials and Methods

In Vivo Perfusion of rIL-3, rGM-CSF, or rEpo. *Escherichia coli* murine rIL-3, prepared as described previously (1), contained <1 ng/ml of LPS. Murine rGM-CSF (4) and human rEpo (5) were kindly provided by Dr. J. J. Mermod, GLAXO S. A., Geneva, Switzerland, and Chugai Pharmaceutical Co., Ltd., Shizuoka, Japan, respectively. Alzet osmotic minipumps (model 2002; Alza Corp., Palo Alto, CA), loaded with rIL-3 (20 μg), rGM-CSF (20 μg), and rEpo (2 μg) in PBS containing 10% (vol/vol) glycerol, or PBS containing 10% glycerol and 10 ng/ml LPS as a control, were placed subcutaneously in 3-4-mo-old (C57BL/10 × BALB/c)F$_1$ (B10 × BALB) mice. The presence of circulating rIL-3 or rGM-CSF in perfused animals was controlled by IL-3- or GM-CSF-specific bioassay, as described previously (6). Epo activity in sera was determined by their ability to maintain the growth of a FDC-P2 cell line, as described (7). The specificity of the bioassays was controlled in the presence of rabbit anti-IL-3 (6), anti-GM-CSF (6), and/or anti-Epo (a kind gift of Dr. S. Kamachi, Chugai Pharmaceutical Co., Ltd.) antibodies.

Anti-MRBC mAb and Hematocrit (Ht) Determination. An IgG1 monoclonal anti-MRBC autoantibody (31-9D) (3) was prepared by cell fusion of NS-2 myeloma cell line and spleen cells from

unmanipulated NZB mice. Culture supernatant concentrated by precipitation in 50% saturated ammonium sulfate, containing ~300 μg 31-9D mAb, was used for intraperitoneal injection. Hts were determined by centrifugation for 5 min at 12,000 rpm of blood samples in heparinized microhematocrit tubes (Clay Adams; Becton Dickinson & Co., Mountain View, CA).

Histopathology. Spleens and livers were obtained at autopsy, processed for histological examination, and stained with hematoxylin and eosin.

Opsonization of MRBC with Anti-MRBC mAb. 5 ml of culture supernatant containing ~100 μg of 31-9D anti-MRBC mAb was incubated with 250 μl of 25% MRBC suspension freshly prepared from BALB/c mice. After an overnight incubation at 4°C, and washing three times with PBS, a 25% suspension of opsonized MRBC in PBS was prepared.

In Vitro Fc Receptor–mediated Phagocytosis. 3 × 10^5 peritoneal cells from unmanipulated BALB/c mice in 3 ml of DME were allowed to adhere to 6-well plates (Falcon Labware, Oxnard, CA) by a 1-h incubation at 37°C. After washing, adherent macrophages were incubated for 3 d in the presence or absence of rIL-3 or rGM-CSF (100 ng/ml/d) in DME supplemented with additional amino acids (6), insulin (15 μg/ml), transferrin (1.5 μg/ml), testosterone (2 mM), linoleic acid (1 μg/ml), 5 × 10^{-5} M 2-ME, and BSA (0.5 mg/ml). Then, 10 μl of 25% opsonized MRBC were added and incubated for 20 min at 37°C. After extensive washings with Hanks' medium, plates were treated with distilled water for 10 s to lyse extracellular MRBC, and isotonicity was quickly restored by adding Hanks' medium. Phagocytic activity was expressed by counting on phase-contrasted photographs the percentage of phagocytic cells and the total number of phagocytosed MRBC per 100 cells.

Results and Discussion

Four groups of (B10 × BALB)F₁ mice received osmotic minipumps placed subcutaneously and loaded with either murine rIL-3 (n = 12), murine rGM-CSF (n = 5), human rEpo (n = 12), or PBS (n = 12) as a control. 3 d later, mice received a single intraperitoneal injection of the lethal dose (300 μg) of monoclonal IgG1 anti-MRBC autoantibody (31-9D). The injection of 31-9D anti-MRBC mAb in control PBS-perfused mice rapidly induced a severe anemia, as documented by a rapid and marked decrease in Ht values (Fig. 1 *A*). By 5 d, all the mice were dead from anemia (Fig. 1 *B*). An identical mortality rate was observed in mice perfused with rGM-CSF (Fig. 1 *B*), although they had somewhat lower Ht values 2 d after the anti-MRBC mAb injection (13.8 ± 0.4%) than did control PBS-treated mice (18.3 ± 3.0%) (p <0.005). Notably, mice perfused with rGM-CSF had detectable levels of circulating GM-CSF (data not shown). In the group of mice perfused with rEpo,
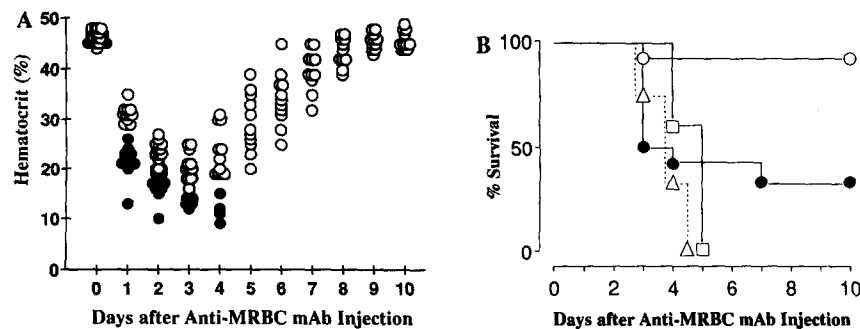

FIGURE 1. (*A*) Ht values of PBS-(●) or rIL-3-(○) perfused mice after the injection of anti-MRBC mAb. (*B*) Survival curve of mice perfused with either rIL-3 (○——○), rGM-CSF (□——□), rEpo (●——●), or PBS (△— — —△) after a single intraperitoneal injection of anti-MRBC mAb.

the anemia after the anti-MRBC mAb administration showed kinetics similar to those of the control group of mice (Ht values: before the mAb injection, 49.3 ± 1.3%; 24 h after the injection, 23.5 ± 4.7%). Although the majority of mice died of acute anemia by 7 d after the anti-MRBC mAb injection, 4 of 12 mice survived and progressively recovered from anemia by 10 d (mean Ht values of surviving mice at 10 d, 44.8 ± 4.5%). It must be stressed that in mice suffering from anemia and not perfused with rEpo, significant activity of Epo was detectable in sera 4 d after the anti-MRBC mAb injection (data not shown). This indicated that the additional exogenous supply of Epo was not sufficient to insure full protection against the most severe form of anemia, presumably because of the limited size of the pool of intermediate and late erythroid precursors responsive to Epo. In contrast, the treatment with rIL-3 almost completely protected the animals from death due to acute anemia (Fig. 1 B). The Ht values of these mice were consistently higher than those of the control group of mice (p <0.001) (Fig. 1 A). Ht values began to increase 4 d after the anti-MRBC mAb injection, and progressively returned to normal levels by 8–10 d. Consequently, all but one mouse recovered from the acute severe form of anemia.

Histological examination revealed remarkable pathological changes in the spleens and livers of the mice that died of anemia. The anti-MRBC mAb injection induced a threefold increase in splenic weight (292 ± 35 mg), as compared with untreated control mice (98 ± 18 mg); this corresponded on a histological section to an enormous accumulation of agglutinated RBC, rendering the splenic architecture hardly recognizable (Fig. 2 A). In the livers, agglutinated RBC accumulated in sinusoids, causing marked necrosis of hepatic parenchymal cells (Fig. 2, C and D). Since erythrophagocytosis by Kupffer cells or by splenic macrophages was hardly detectable, the major cause of anemia was apparently the marked sequestration of agglutinated RBC, mostly in spleen and liver. The finding that erythrophagocytosis was limited can be explained by the fact that the mAb used in the present study is of the IgG1 subclass, and that the affinity of Fc receptor for IgG1/IgG2b is known to be very weak (8). In contrast, the mice that recovered from the anemia due to the perfusion of rIL-3 did not show splenic accumulation of agglutinated RBC (Fig. 2 B), although their spleens were enlarged as a result of an expansion of hemopoietic progenitors stimulated by rIL-3 (2, 3). Similarly, the histological appearance of their livers was essentially normal (Fig. 2 E), except for the presence of a number of extramedullary hemopoietic foci containing mostly cells from the erythroid and myeloid lineages. In contrast, the spleens and livers of the mice that recovered from anemia after the rEpo perfusion showed areas of fibrosis surrounding necrotic foci (Fig. 2, F, G, and H).

Since mice perfused with rIL-3 completely recovered from anemia without significant fibrosis in spleens and livers, one of the beneficial effects of rIL-3 on animals' survival may be related to its enhancing activity of phagocytosis, as shown previously in mice injected with rIL-3 (2). To determine whether rIL-3 was indeed able to stimulate macrophages to increase their phagocytic activity of MRBC coated with 31-9D anti-MRBC mAb, resident peritoneal macrophages from BALB/c mice were first stimulated in vitro with either rIL-3 or rGM-CSF for 3 d, and then their phagocytic activity of anti-MRBC mAb-coated MRBC was assessed. Macrophages incubated with rIL-3 or rGM-CSF exhibited higher percentages of phagocytic cells (82 and 89%, respectively) and phagocytosed larger numbers of antibody-coated MRBC (303 and 375 MRBC per 100 macrophages, respectively) than did macro-
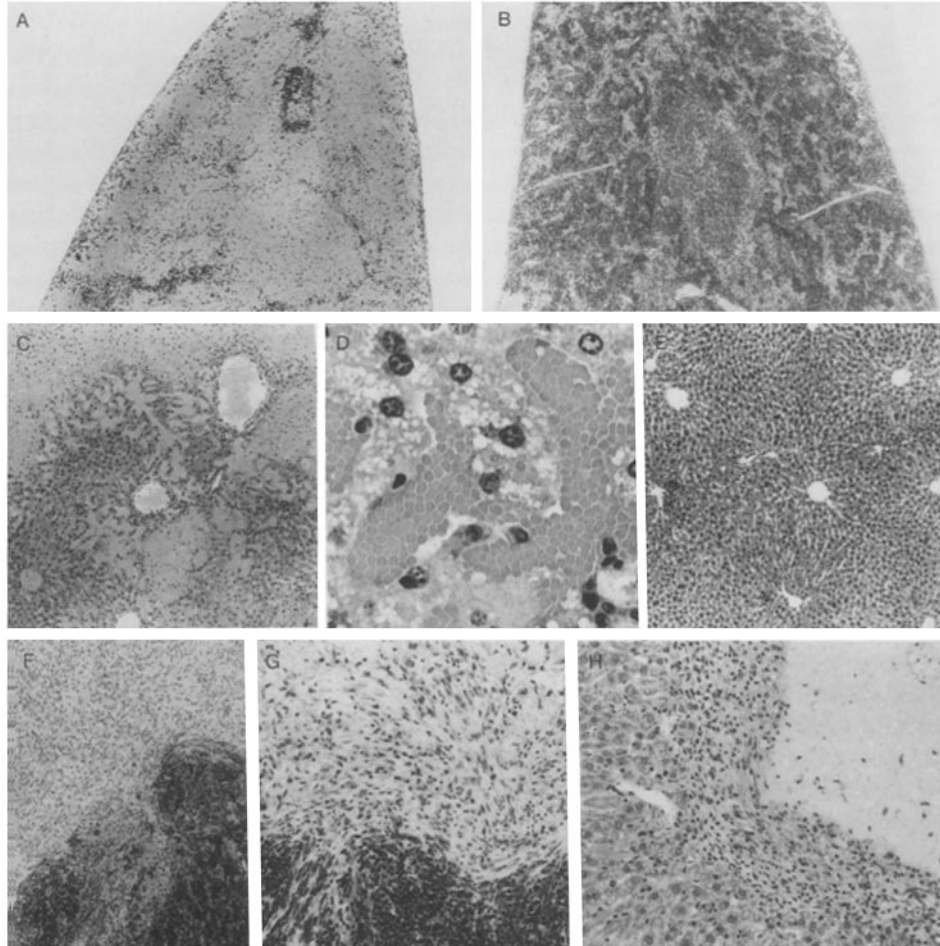
FIGURE 2. (A, C, and D) Representative histological appearance of spleen and liver from control PBS-perfused mice that died of acute anemia 4 d after the injection of anti-MRBC mAb. Note an enormous accumulation of agglutinated RBC in entire spleen, which does not allow it to recognize splenic structures (A; HE, ×40), and marked necrosis of hepatic parenchymal cells (C; HE, ×40) secondary to the accumulation of agglutinated RBC in sinusoids of liver (D; HE, ×400). (B and E) Representative histological appearance of spleen and liver from rIL-3-perfused mice that recovered from acute anemia induced by anti-MRBC mAb (10 d after the anti-MRBC mAb injection). Note complete absence of accumulation of agglutinated RBC in spleen (B; HE, × 40) and liver (E; HE, × 40). (F, G, and H) Representative histological appearance of spleen and liver from rEpo-perfused mice that recovered from acute anemia induced by anti-MRBC mAb (10 d after the anti-MRBC mAb injection). Note the presence of fibrosis in spleen (F and G; HE ×40 and ×100, respectively) and around necrotic focus in liver (H; HE, ×100).

phages in the control unstimulated culture (the percent of phagocytic cells was 24%, and the number of opsonized MRBC phagocytosed by 100 macrophages was 107). This enhanced phagocytic activity of macrophages was mediated by Fcγ receptor, as shown by the complete inhibition in the presence of a rat anti-Fcγ receptor mAb (2.4G2) (9) (data not shown).

The data presented here have demonstrated that rIL-3 perfusion is able to protect animals from death due to an acute severe form of anemia induced by a monoclonal IgG1 anti-MRBC autoantibody, while only partial protection (one third of the cases) by rEpo and little effect by rGM-CSF are observed. This protection could be related to several effects of rIL-3 perfusion on hemopoietic cells. First, marked expansion of early hemopoietic progenitors by rIL-3 treatment increases the accumulation of intermediate and late erythroid progenitors responsive to Epo (1, 2). Because anemic mice rapidly produce large amounts of Epo, as documented by the presence of high levels of circulating Epo, the increase in Epo-responsive precursors by the IL-3 per-fusion is an important factor. The failure of rEpo and the limited activity of murine rGM-CSF to promote the proliferation and differentiation of early precursors of the erythroid lineage (10) would explain the only partial protection exerted by rEpo and the absence of protective effect by rGM-CSF. Second, since severe liver damage due to an enormous accumulation of agglutinated RBC in sinusoids can also con-tribute to the animals' death, the enhancement of macrophage phagocytic activity by IL-3, as shown by others (2) and in our present study, may lead to efficient elimi-nation of agglutinated MRBC by Kupffer cells or splenic macrophages. The pro-motion of the proliferation and differentiation of monocyte/macrophage lineage by rIL-3 perfusion (1, 2) can further exhibit an additive effect on the elimination of agglutinated MRBC from livers and spleens. The absence of these activities, there-fore preventing a rapid removal of agglutinated RBC, would account for the devel-opment of fibrosis around necrotic tissues observed in spleens and livers of surviving mice perfused with rEpo. Clearly, these effects on macrophages are by themselves insufficient to insure protection, as shown by the inefficiency of rGM-CSF, which has similar effects on the monocyte/macrophage lineage (10, 11). Finally, one may wonder whether the perfusion with rIL-3 or rEpo starting before the anti-MRBC mAb injection allows a rapid rise in RBC that absorb some of anti-MRBC mAb, thereby markedly diminishing consequent accumulation of agglutinated RBC in spleens and livers. The successful treatment of acute severe anemia by rIL-3 sug-gests that such a therapeutic approach might be considered in combination with Epo in some patients with acute severe form of hemolytic anemia, in whom the dis-order is refractory to standard forms of therapy.

Summary

We have evaluated the therapeutic activity of rIL-3, in comparison with recom-binant granulocyte-macrophage CSF (rGM-CSF) and recombinant erythropoietin (rEpo), on a lethal form of acute anemia induced by a single injection of a mono-clonal IgG1 anti-mouse RBC (MRBC) autoantibody. Continuous perfusion of rIL-3 before the administration of anti-MRBC mAb prevented animals from the death due to anemia with a rapid recovery in >90% of the cases, while only partial protec-tion (one third of the cases) was obtained by rEpo perfusion, and no protection by rGM-CSF. Since the anti-MRBC mAb induced a marked agglutination of RBC in spleens and livers, and subsequent hemodynamic failure may be an additional contributing factor to the animals' death, the activation of Fcγ receptor–dependent phagocytosis by rIL-3, as well as the increased number of monocytes/macrophages resulting from rIL-3 perfusion, may also facilitate rapid elimination of these agglu-tinated RBC, resulting in the further amelioration of the animals' survival. Our results

suggest that the therapeutic effect of rIL-3 on anti-MRBC autoantibody–induced anemia is achieved by: (a) its activity to promote the growth and differentiation of erythroid progenitors responsive to Epo and of monocyte/macrophage lineage; and (b) its activity to enhance the phagocytic activity of macrophages to efficiently eliminate agglutinated RBC in spleens and livers.

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