were maintained in air at 5°C and 100 percent relative humidity.
7. G. K. Noble and M. K. Brady, *Zoologica* **11**, 89 (1933).
8. Eggs were covered to a depth of 3 cm with water equilibrated with different mixtures of $N_2$ and $O_2$. The $PO_2$ was decreased approximately 15 torr at each time period. Vials were identical to those described in Table 1.
9. Reported temperature optima for amphibian hatching enzymes are near 30°C.
10. H. Sugawara, *J. Fac. Sci. Imp. Univ. Tokyo Sect. 4* **6**, 109 (1943).
11. J. Ishida, *Annot. Zool. Jpn.* **22**, 137 (1944); K. Yamagami, *Comp. Biochem. Physiol.* **46B**, 603 (1973).
12. B. F. Edwards, W. R. Allen, D. Barrett, *Arch. Biochem. Biophys.* **182**, 696 (1977).
13. F. H. Pough, *Science* **192**, 68 (1976); T. J. C. Beebee and J. R. Griffin, *J. Zool.* **181**, 341 (1977); P. A. Saber and W. A. Dunson, *J. Exp. Zool.* **204**, 33 (1978).
14. F. R. Hayes, *J. Exp. Zool.* **89**, 357 (1942).
15. C. Katagiri, *J. Fac. Sci, Hakkaido Univ. Ser. 6 Zool.* **18**, 461 (1973); *J. Exp. Zool.* **193**, 109 (1975); K. Takeuchi, H. Yokosawa, M. Hoshi, *Eur. J. Biochem.* **100**, 257 (1979); U. A. Urch and J. L. Hendrick, *J. Supramol. Struct.* **15**, 111 (1981).
16. In initial studies we also induced *Ambystoma texanum* and *Rana palustris* to hatch in water equilibrated with nitrogen.
17. J. A. Moore, *Am. Nat.* **74**, 89 (1940); T. Yani, *Zool. Mag. (Tokyo)* **61**, 164 (1952).
18. R. Milkman, *Biol. Bull. (Wood's Hole, Mass.)* **107**, 300 (1954); D. F. Alderdice, W. P. Wickett, J. R. Brett, *J. Fish. Res. Board Can.* **15**, 229 (1958); K. Hamdorf, *Z. Vgl. Physiol.* **44**, 523 (1961); N. Peters, *Int. Rev. Gesamten Hydrobiol.* **48**, 257 (1963); N. V. Kotlyarevskaya, *Dokl. Biol. Sci.* **177**, 746 (1967).
19. L. Dimichele and M. H. Taylor, *J. Exp. Zool.* **214**, 181 (1980).
20. H. E. Hagenmaier, *Experientia* **28**, 1214 (1972); K. Yamagami, *Am. Zool.* **21**, 459 (1981).
21. C. M. Gjullin, C. D. Hegarty, W. B. Bollen, *J. Cell. Comp. Physiol.* **17**, 193 (1941); J. J. Kardatzke, *Ann. Entomol. Soc. Am.* **72**, 559 (1979).
22. R. H. Brewer, *Physiol. Zool.* **37**, 1 (1964).
23. J. S. Clegg, *J. Exp. Biol.* **41**, 879 (1964); F. Horne, *Ecology* **48**, 472 (1967); R. E. Hall and L. J. MacDonald, *Hydrobiologia* **46**, 369 (1975); G. R. Daborn, *Can. J. Zool.* **55**, 161 (1977).
24. J. F. Mueller, *J. Parasitol.* **45**, 353 (1959); S. MacDonald and C. Combes, *Chronobiologia* **5**, 272 (1978).
25. W. G. Moore, *Ecology* **44**, 131 (1963); J. A. Bishop, *J. Anim. Ecol.* **36**, 599 (1967); L. R. Brown and L. H. Carpelan, *Ecology* **52**, 41 (1971); S. Kashara, T. Oube, M. Kamigakli, *Mar. Biol. (New York)* **31**, 31 (1975).
26. Supported by a University of Kentucky Graduate School Fellowship (to J.W.P.) and NIH Biomedical grant 2-SO7-RR07114-13 (to J.J.J.). We thank Jeanne Just, David Prior, and Gary Uglem for constructive criticisms.

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## Transplantation of Leukemic Bone Marrow Treated with Cytotoxic Antileukemic Antibodies and Complement

Abstract. *The ability of antiserum against murine L1210 leukemia to remove residual leukemia cells from murine bone marrow was investigated. Leukemic marrow was treated in vitro with antiserum and complement and used to hematologically reconstitute mice that had been irradiated with doses lethal to bone marrow. Following infusion of treated leukemic marrow, normal marrow returned without evidence of leukemia. More than 90 percent of the animals have survived for 11 months without untoward effects, suggesting that the technique may be of use in the treatment of acute leukemia in humans.*

Transplantation of allogeneic bone marrow is often used to treat leukemic patients whose disease has resisted standard therapy (*1, 2*). In this approach, leukemic patients are given chemotherapy and total body radiation to destroy the bone marrow and then receive an intravenous infusion of marrow from a histocompatible donor. The procedure has met with some success but also has certain limitations (*3*). Many patients lack a histocompatible bone marrow donor, and there is significant morbidity from graft-versus-host disease.

An alternative approach involves the reinfusion, following marrow ablation, of autologous bone marrow harvested during a remission (*4*). This method eliminates the need for histocompatible donors and the problem of graft-versus-host disease. However, preliminary attempts with this approach have been disappointing because leukemia has recurred coincident with or before the return of normal hematopoietic activity (*4*). This is presumably due to a reinfusion of viable leukemia cells present in the marrow obtained during "remission" or to a failure of the conditioning regimen. To avoid the problem of infusing leukemia cells, in vitro elimination of leukemia cells from so-called "remission" bone marrow by using specific antiserum to leukemia has been considered (*5*). This approach is now being attempted in some patients (*6*).

In this study we demonstrate that specific antiserum can eliminate leukemia cells from murine bone marrow in vitro and that long-term disease-free survival is possible following reconstitution with the treated marrow.

Antiserum to murine L1210 leukemia (*7*) was prepared in accordance with a modification of a previously described technique (*8*). New Zealand White rabbits were injected intravenously with $2 \times 10^8$ viable L1210 cells on eight separate occasions over a 4-week period. Prior to immunization the cells were sedimented through a Ficoll-Hypaque density gradient to remove contaminating red cells and resuspended in 2 ml of undiluted rabbit antiserum to DBA/2 thymocytes. Blood was collected 1 week after the eighth injection. The serum obtained was adsorbed at 37°C against erythrocytes, thymocytes, and splenocytes from C57B1/6 and DBA/2 mice. A 1:4 dilution of the adsorbed antiserum produced 100 percent lysis of leukemia cells in a complement-dependent in vitro assay of cytotoxicity (*8*).

In the first experiment we determined the dose of L1210 leukemia cells required to kill normal DBA/2 mice. Intraperitoneal injection of as few as ten viable cells killed more than 60 percent of the mice within 21 days (Fig. 1A). This indicated that if ten or more viable leukemic cells remained in the leukemic bone marrow following treatment in vitro, the animals reconstituted with such marrow would succumb to recurrent leukemia.

The next experiment was designed to determine the cytotoxic efficacy of the antiserum and normal rabbit complement. Some $10^5$ viable cells were incubated with antiserum, complement, or both for 60 minutes at 22°C and then injected intraperitoneally into healthy DBA/2 mice. No deaths occurred in the mice injected with leukemia cells incubated with antiserum and complement (Fig. 1B). In contrast, 100 percent mortality was observed in animals injected with untreated cells or with cells that had been incubated with antiserum or complement alone.

From these studies it was concluded that the animals were unable to survive injections of leukemia cells coated only with heterologous antiserum. Presumably the host was unable to recognize antibody-coated cells as foreign. However, since all animals survived free of leukemia when injected with the antibody and complement-treated cells, our transplantation experiments were planned to treat leukemic bone marrow in vitro with both these components. These experiments were performed as follows.

Bone marrow was obtained from the femurs of female DBA/2 mice. The marrow to be transplanted into one group of mice was treated with an equal volume of hypotonic buffer containing EDTA, $NH_4Cl$, and $KHCO_3$. Viable L1210 cells were added to yield a final concentration of 2.5 percent L1210 cells in the marrow preparation. Following sedimentation the supernatant was removed and the leukemic marrow pellet was incubated at 22°C for 30 minutes with undiluted, adsorbed, L1210-specific antiserum. Normal rabbit serum as a source of complement was then added in a concentration of 2.5:1 and incubated with the marrow at 22°C for 60 minutes. The incubation mixture was shaken frequently. The treated marrow was then sedimented,


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the serum was removed, and the pellet was resuspended in phosphate-buffered saline. Male DBA/2 mice were exposed to total body irradiation with $Ce^{137}$ (129 roentgens per minute; total dose, 875 R) to ablate their bone marrow. Twenty-four hours later the mice were injected intravenously with the treated marrow ($2 \times 10^8$ nucleated cells per kilogram of body weight).

As shown in Fig. 1C, 90 percent of the irradiated mice given marrow incubated with antiserum and complement survived normally. There were no survivors among mice receiving marrow incubated with antiserum or complement alone.

These studies demonstrate that in vitro treatment of leukemic bone marrow with specific antiserum to leukemia successfully eliminates leukemia cells. Since the injection of as few as ten L1210 cells kills a majority of normal mice, the technique reported here was more than 99.9 percent effective in eliminating leukemia cells from the marrow, which contained a minimum of $10^5$ L1210 leukemia cells.

A crucial step in the treatment process is the preparation of the leukemic marrow in hypotonic buffer. This prevents the clumping of marrow in vitro. There were no survivors among mice transplanted with bone marrow that was incubated with the antiserum and complement but not given the initial treatment with hypotonic buffer (Fig. 1D). Clumping of the marrow may prevent uniform exposure of L1210 cells to cytotoxic antiserum and complement. Injections of leukemia cells treated with the hypotonic buffer resulted in survival curves identical to those shown in Fig. 1, A and B, indicating that the hypotonic buffer has no antileukemic effect.

Two survivors of the transplantation experiments died from a Sendai virus infection 2 months after hematopoietic reconstitution. Two others died 10 months later and one died at 11 months. None of these mice showed evidence of leukemia. The remaining 13 mice are alive and healthy after 11 months.

For 60 years investigators have been studying serotherapy of malignant diseases (*9*). However, the administration of tumor-specific antiserum alone has not been demonstrated to prevent the growth of a spontaneous malignancy in any animal system (*10*) and in some situations has been associated with an enhancement of tumor growth (*11*). The systemic use of tumor-specific antiserum has also failed to cure any human malignancy (*9*). The ineffectiveness of systemically administered antiserum against tumors may involve such considerations as the degree of tumor burden, the problem of delivery to the tumor site, and the difficulty associated with the injection of foreign proteins. A recent unsuccessful attempt at systemic serotherapy of acute lymphoblastic leukemia in man was associated with antigenic modulation, another potential problem of this approach. Nevertheless, the systemic use of tumor-specific serums or monoclonal antibodies may yet provide an important adjunct to currently accepted forms of treatment (*12, 13*).

The success of our experiments suggests that immunotherapy of bone marrow obtained during remission may be effective in treating patients with acute leukemia. We hope that these results will stimulate the development of cytotoxic monoclonal antibodies against leukemia that are nontoxic to human bone marrow and appropriate for this type of immunotherapy.

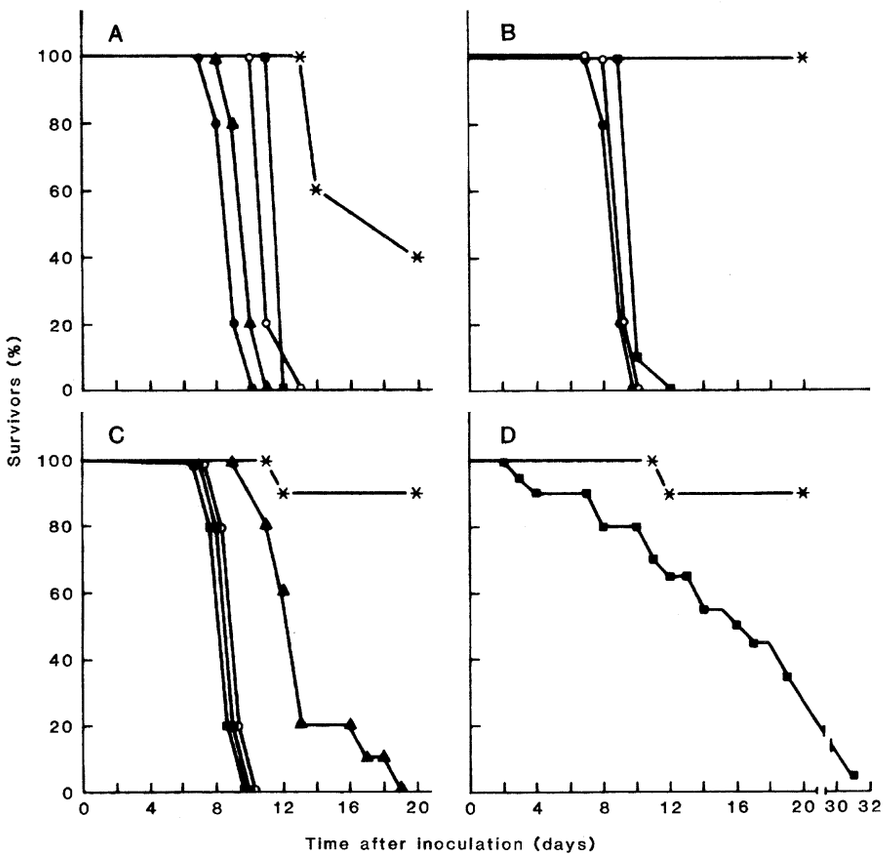


Fig. 1. (A) Results of inoculating male DBA/2N mice intraperitoneally with viable L1210 leukemia cells on day 0. (●) Group 1, 50 mice given $10^5$ cells; (▲) group 2, 20 mice given $10^4$ cells; (○) group 3, 20 mice given $10^3$ cells; (■) group 4, 20 mice given $10^2$ cells; and (*) group 5, 20 mice given ten cells. (B) Results of inoculating mice intraperitoneally with $10^5$ L1210 leukemia cells treated in various ways. (●) Group 1, ten mice given untreated cells; (○) group 2, ten mice given cells treated for 60 minutes with normal rabbit complement; (■) group 3, 20 mice given cells incubated with antiserum for 30 minutes; and (*) group 4, 20 mice given cells treated with antiserum for 30 minutes and then with complement for 60 minutes. (C) Results of irradiating mice and then reconstituting them hematologically in various ways. (▲) Group 1, 20 untreated (control) mice receiving only irradiation; (●) group 2, ten mice inoculated intravenously with syngeneic bone marrow that contained 2.5 percent viable L1210 cells and that had been treated with antiserum for 30 minutes before inoculation. Group 3 (○) was same as group 2 except that the leukemic marrow had been incubated with complement for 60 minutes instead of with antiserum for 30 minutes. (■) Group 4, ten mice inoculated intravenously with leukemic marrow that had not been treated in vitro; and (*) group 5, 20 mice inoculated intravenously with syngeneic marrow that contained 2.5 percent leukemia cells and that had been treated with antiserum and complement before inoculation. The treated leukemic marrow given to groups 2, 3, and 5 was prepared in hypotonic buffer. (D) Results of irradiating mice on day 0 and then inoculating them intravenously with syngeneic marrow that contained 2.5 percent leukemia cells and that had been treated with antiserum and complement. Group 1 (*) comprised 20 mice receiving marrow prepared in hypotonic buffer and group 2 (■) comprised 20 mice receiving marrow not prepared in hypotonic buffer.


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**References and Notes**

1. E. D. Thomas *et al.*, *Blood* **49**, 511 (1977); E. D. Thomas *et al.*, *ibid.* **54**, 468 (1979); J. A. Hansen, R. A. Clift, E. D. Thomas, C. D. Buckner, R. Storb, E. R. Giblett, *N. Engl. J. Med.* **303**, 565 (1980).
2. P. L. Weiden, N. Flournoy, J. E. Sanders, K. M. Sullivan, E. D. Thomas, *Transplant. Proc.* **13**, 248 (1981).
3. K. Sullivan, P. Weiden, N. Flournoy, R. Storb, E. D. Thomas, *Proc. Am. Soc. Clin. Oncol.* **22**, 477 (1981); P. L. Weiden, K. M. Sullivan, N. Flournoy, R. Storb, E. D. Thomas, *N. Engl. J. Med.* **304**, 1529 (1981); D. A. Vallera, C. C. B. Soderling, G. J. Carlson, J. H. Kersey, *Transplantation* **31**, 218 (1981).
4. P. R. Graze and R. P. Gale, *Transplant. Proc.* **10**, 177 (1978).
5. J. R. Wells, R. Billing, P. Herzog, S. A. Feig, R. P. Gale, P. Terasaki, M. J. Cline, *Exp. Hematol.* **7**, 164 (1979); R. Billing, J. R. Wells, R. P. Gale, K. Foon, M. Linker-Israel, C. Deng, P. I. Terasaki, M. J. Cline, *Biology of Bone Marrow Transplantation* (Academic Press, New York, 1980), pp. 167–173; J. R. Wells, A. Sullivan, M. J. Cline, *Cryobiology* **16**, 201 (1979); M. J. Cline, D. W. Golde, R. J. Billing, J. E. Groopman, J. Zighelboim, R. P. Gale, *Ann. Intern. Med.* **91**, 758 (1979); R. J. Maas *et al.*, *Blut* **40**, 387 (1980); B. Netzel, H. Rodt, R. J. Haas, H. J. Kolb, S. Thierfelder, *Lancet* **1980-I**, 1330 (1980); A. Ziegler, *Blut* **41**, 1 (1980).
6. R. Billing and S. E. Sallan, personal communication.
7. The L1210 murine leukemia was derived from the original methylcholanthrene-induced line cryopreserved in the tumor bank of the Division of Cancer Treatment, National Cancer Institute (W. L. Grant, personal communication).
8. A. E. Reif and C. H. Kim, *Nature (London)* **223**, 1377 (1969); P. J. Smith, C. M. Robinson, A. E. Reif, *Cancer Res.* **34**, 169 (1974); A. E. Reif, C. M. Robinson, P. J. Smith, *Ann. N.Y. Acad. Sci.* **277**, 647 (1976).
9. G. Lindstrom, *Acta Med. Scand. Suppl.* **22**, 1 (1927); S. Thierfelder, H. Rodt, B. Netzel, *Transplantation* **23**, 459 (1977); B. Netzel, H. Rodt, B. Lau, E. Thiel, R. J. Haas, P. Dormer, S. Thierfelder, *ibid.* **26**, 157 (1978); I. D. Bernstein, M. R. Tam, R. C. Nowinski, *Science* **207**, 68 (1980); P. W. Wright and I. D. Bernstein, *Prog. Exp. Tumor Res.* **25**, 140 (1980); R. A. Miller, D. G. Maloney, J. McKillop, R. Levy, *Blood* **58**, 78 (1981).
10. A. E. Reif, R. W. Li, C. M. Robinson, *Cancer Treat. Rep.* **61**, 1499 (1977); J. S. Economou, *Surgery* **87**, 190 (1980).
11. G. A. Carlson and G. Terres, *J. Immunol.* **121**, 1752 (1978).
12. R. Billing, J. Minowada, M. Cline, B. Clark, K. Lee, *J. Natl. Cancer Inst.* **61**, 423 (1978); E. Thiel, H. Rodt, B. Netzel, D. Huhn, G. F. Wundisch, R. J. Haas, C. H. Bender-Gotze, S. Thierfelder, *Blut* **36**, 363 (1978); B. Netzel, H. Rodt, E. Thiel, R. J. Haas, S. Thierfelder, *Klin. Paediatr.* **191**, 175 (1979); W. W. Young, Jr., and S. Hakomori, *Science* **211**, 487 (1981).
13. J. Ritz, J. M. Pesando, S. E. Sallan, L. A. Clavell, J. Notis-McConarty, P. Rosenthal, S. F. Schlossman, *Blood* **58**, 141 (1981).


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# An Enzymatic System for Removing Heparin in Extracorporeal Therapy


Abstract. *The need to fully heparinize patients undergoing extracorporeal therapy often leads to hemorrhagic complications. To enable heparinization of only the extracorporeal circuit, a blood filter containing immobilized heparinase was developed. This filter degraded 99 percent of heparin's anticoagulant activity within minutes in both canine and human blood.*


Extracorporeal devices perfused with blood, such as the artificial kidney and the pump-oxygenator, have been an important part of the clinician's armamentarium for many years. These devices all rely on the patient being heparinized to prevent clotting in the device. However, heparinization of the patient often leads to hemorrhagic complications (*1*, *2*). With the prospect of longer perfusion times with machines such as the membrane oxygenator (*3*), the drawbacks of systemic heparinization are multiplied. A number of approaches have been tested to solve this problem. They include (i) administering protamine to neutralize heparin (*4*), (ii) using antithrombotic drugs other than heparin (*5*), (iii) bonding heparin, albumin, or fibrinolytic enzymes to the extracorporeal device (*6*), and (iv) developing new blood-compatible materials for construction of the device (*7*). Although these approaches have led to some improvements, control of the heparin concentration in the blood remains a serious problem.

We suggest a novel approach that would permit full heparinization of blood entering any extracorporeal device but which would enable enzymatic elimination of the heparin before the blood returned to the patient. This approach consists of placing a blood filter containing immobilized heparinase—which degrades heparin into small polysaccharides—at the effluent of the extracorporeal device. Initial studies reported here to test the feasibility of this approach show that a conventional blood transfusion filter containing immobilized heparinase can remove the anticoagulant activity of clinically used amounts of heparin in several minutes in human blood and in dogs.

Heparinase was produced from *Flavobacterium heparinum* (*8*) and purified by cell sonication, nucleic acid precipitation with protamine sulfate, and hydroxylapatite chromatography (*9*). At this stage of purification, heparinase degrades 150 mg of heparin per milligram of protein per hour and is catalytically pure (free from contaminating sulfatases and glucuronidases) (*10*). Heparinase was covalently bound to Sepharose and washed to remove noncovalently bound enzyme by a variation (*9*) of the procedure of March *et al.* (*11*). There was no detectable leaching, as judged by protein concentration (*12*), of heparinase from the support over a 1-month period at 25°C.

Immobilization increases the stability of heparinase: at 4°, 25°, and 37°C the immobilized enzyme has half-lives of 5000, 1000, and 15 hours, respectively, compared with the native enzyme, which has half-lives of 125 and 30 hours and 1 hour at the same temperatures.

Active, immobilized heparinase and a control consisting of thermally inactivated, immobilized heparinase were loaded into two columns with a 1.5-ml bed volume. Heparin solutions were passed through both columns in stepwise fashion at concentrations of 15 to 75 μg/ml (clinically used heparin concentrations, having a specific activity of 0.15 U/μg, range from 5 to 25 μg/ml) (Fig. 1). As the heparin level in the input solutions increased, the difference in the heparin recovered at the outlets of the columns increased (Fig. 1). Even at 75 μg/ml, the heparin was largely removed by the column containing active heparinase, while the control column had no effect.

At flow rates above 1 ml/min the Sepharose packed tightly, preventing flow. Therefore we used a fluidized bed of Sepharose, obtained by pumping Sepha-

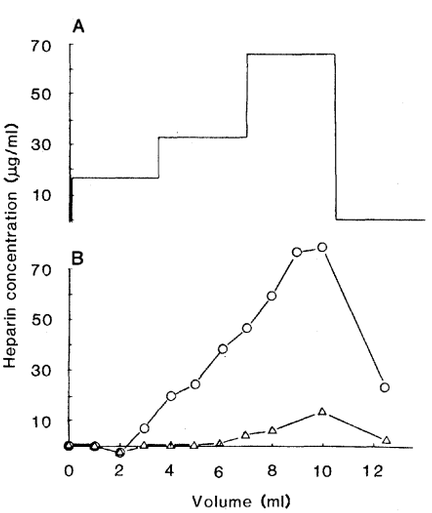


Fig. 1. (A) Input concentrations of heparin to both active and inactive columns, showing stepwise increases from 15 to 75 μg/ml. (B) Output concentrations of heparin from the column containing active heparinase (△) and the column containing heat-inactivated heparinase (○). Solutions of bovine serum albumin (60 mg/ml), salts, and heparin were passed through the columns (diameter, 1 cm) at a flow rate of 0.5 ml/min. The inactive heparinase was prepared by heating a suspension of active Sepharose-heparinase at 100°C for 30 minutes. The physiological concentrations of the nonheparin species in blood were used. Heparin concentration was measured by the azure assay (*8*, *17*).



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