References and Notes

1. I. Schecter and A. Berger, *Biochem. Biophys. Res. Commun.* **27**, 157 (1967).
2. N. Abramowitz, I. Schechter, A. Berger, *ibid.* **29**, 862 (1967).
3. H. M. Lazarus, M. B. Sporn, D. F. Bradley, *Proc. Nat. Acad. Sci. U.S.* **60**, 1503 (1968).
4. P. Cuatrecasas, S. Fuchs, C. B. Anfinsen, *J. Biol. Chem.* **242**, 1541 (1967).
5. P. Cuatrecasas, H. Taniuchi, C. B. Anfinsen, *Brookhaven Symp. Quant. Biol.*, in press.
6. M. Alexander, L. A. Heppel, J. Hurwitz, *J. Biol. Chem.* **236**, 3014 (1961).
7. E. Sulkowski and M. Laskowski, Sr., *ibid.* **237**, 2620 (1962).
8. L. Morávek, C. B. Anfinsen, J. Cone, H. Taniuchi, in preparation.
9. S. Fuchs, P. Cuatrecasas, C. B. Anfinsen, *J. Biol. Chem.* **242**, 4768 (1967).
10. P. Cuatrecasas, M. Wilchek, C. B. Anfinsen, in preparation.
11. M. Dixon, *Biochem. J.* **55**, 170 (1953).
12. G. H. Beaven, E. R. Holiday, E. A. Johnson, in *The Nucleic Acids: Chemistry and Biology*, E. Chargaff and J. Davidson, Eds. (Academic Press, New York, 1955), vol. 1, p. 509.
13. F. A. Cotton, D. Richardson, J. S. Richardson, E. E. Hazen, A. Arnone, personal communication.
14. P. Cuatrecasas, S. Fuchs, C. B. Anfinsen, *J. Biol. Chem.* **242**, 3063 (1967).
15. K. K. Reddi, *Biochim. Biophys. Acta* **33**, 132 (1959).
16. W. Roberts and C. Dekker, *Fed. Proc.* **19**, 318 (1960).

* Present address: Weizmann Institute of Science, Rehovoth, Israel.

30 September 1968; revised 14 November 1968 ■

Warfarin Treatment of Mice Bearing Autochthonous Tumors: Effect on Spontaneous Metastases

Abstract. *Long-term oral administration of sodium warfarin significantly reduced the incidence of spontaneous metastases in the lungs from 83 percent in controls to 8 percent in treated C57/BL/6N mice. The size and weight of primary tumors in mice treated with warfarin were less than in control mice. Length of survival was unaffected.*

The mechanism of blood coagulation, and in particular of fibrin deposition, is important in the growth of a primary tumor and its metastatic spread. O'Meara and Jackson (*1*) have noted deposition of fibrin in a wide variety of tumors, particularly at the invading periphery, and they felt that fibrin was an important lattice for tumor growth. Human cancer cells contain an agent [cancer coagulative factor (*2*)] that induces fibrin formation and is thought to resemble thromboplastins (*3*). The action of this factor can be blocked in humans and rabbits by dicoumarol and similar coumarin anticoagulants (*3*). The formation of an enveloping microthrombus around embolic tumor cells is a decisive part of the mechanism of endothelial adherence and penetration (*4*). Adequate anticoagulation or fibrinolysis with numerous agents significantly reduces the incidence of metastases (*4–7*), whereas agents that increase the ability of blood to coagulate increase this incidence (*4, 5*). A clinical study by Michaels suggested that treatment with anticoagulants might similarly affect the metastatic spread of human tumors (*8*). In recent evaluations of protamine (*9*), plasmin (*10*), and protease (*11*) in patients with cancer, investigators attempted to impair tumor growth by altering the mechanisms of coagulation or fibrinolysis, but the results have been inconclusive.

Most studies from which a relationship between coagulation, fibrin, and the growth and spread of tumors has been established have been performed with transplanted tumors in inbred animals. There have been valid questions regarding the degree to which the interaction of host and transplanted tumor resembles the relation of patient and autochthonous tumor (*12*). For this reason we have studied the effect of long-term treatment with sodium warfarin on the incidence of spontaneous metastatic spread from autochthonous tumors to see if the suppressive effect found with transplanted tumors would persist.

Autochthonous tumors were induced in 50 female C57/BL/6N mice, 6 to 8 weeks old, by the injection intramuscularly in the right thigh of 0.1 ml of 3-methylcholanthrene (1 percent) in sesame seed oil. Those mice (25) in which the injected thigh enlarged two to three times between the 8th and 10th weeks after injection were alternately placed in the control or treated group. Although nearly all 50 mice would, over several more months, develop tumors, those with tumors arising in this early period were selected for study because growth and metastatic behavior of these tumors is more uniform. Sodium warfarin (9.10 mg/liter) was added to the drinking water of treated mice. In mice with tumors, this dosage is adequate to maintain a prothrombin time two to three times the normal average value over many weeks (*7*). The warfarin solution was changed every 3rd or 4th day. All mice were kept under observation until death, and their lungs were examined macroscopically for metastases. India ink, used as the stain, differentially colors normal lung and tumor tissue (*13*). Because the tumor frequently was larger than the mouse at death and because it could not be cleanly dissected free, the entire carcass was weighed.

All mice had progressive enlargement of the injected thigh to beyond five times the normal size. Histologic examination revealed that the tumors were poorly differentiated sarcomas, quite uniform in pattern. Frequently, the tumor was larger than the host mouse at the time of death. In our laboratory, the average weight of 6-month old C57/BL/6N mice, the age at which the tumor-bearing mice died, is 18.4 g. The average weight of the tumor-bearing carcass of the control mice was 34.5 g (ranging from 25 to 45 g); the average weight of treated mice was 24.5 g (ranging from 21 to 30 g). The primary tumors of mice treated with warfarin, although large, never reached the huge proportions of tumors in controls.

The mean survival time of control mice after injection was 130 days (range, 123 to 148 days); that of the mice treated with warfarin was 138 days (range, 124 to 158 days). Examination of warfarin treated mice shortly after death frequently showed hemorrhage within the large primary tumors.

At death, ten of the twelve control mice (83 percent) had pulmonary metastatic lesions, and in six of the ten the lesions were too numerous and confluent to permit accurate counting and sizing. Three of these six mice had metastases to inguinal and pelvic lymph nodes as well. Only one of 13 mice treated with warfarin (8 percent) had metastases at death and this was a single lesion. This difference in incidence is significant statistically at P less than .005 by the χ^2 test.

The relation between host and tumor is similar in autochthonous tumors in highly inbred strains of mice and in tumors in man. Transplanted tumors, however, have many possible factors of variance, as discussed by Klein (*12*), making the extrapolation of results from such studies to the clinical situation more hazardous than the obvious host differences alone would imply. The interactions of host and tumor, of decisive importance, may be different. Yet, because transplanted tumors are so much easier to use, studies with

autochthonous tumors are few. The report by Thornes (*3*) of decreased motility and frequent cell death of V_2 carcinoma cells, observed in rabbits treated with dicumerol (*3*), may have relevance to the smaller primary tumors and decreased metastatic spread seen in our study.

JAMES J. RYAN
ALFRED S. KETCHAM
HILDA WEXLER

National Cancer Institute, Bethesda, Maryland 20014

References and Notes

1. R. O'Meara and R. Jackson, *Irish J. Med. Sci.* **391**, 327 (1958).
2. R. O'Meara, *ibid.* **394**, 474 (1958).
3. R. Thornes, in *Endogenous Factors Influencing Host-Tumor Balance*, R. Wissler, T. Dao, S. Wood, Ed. (Univ. of Chicago Press, Chicago, 1966), p. 255.
4. S. Wood, E. Holyoke, J. Yardley, *Proc. Can. Cancer Res. Conf.* **4**, 167 (1961).
5. E. Cliffton and D. Agostino, *Vasc. Dis.* **2**, 43 (1965).
6. D. Agostino, C. Cliffton, H. Cirolami, *Cancer* **19**, 284 (1966).
7. J. Ryan, A. Ketcham, H. Wexler, *Ann. Surg.* **168**, 163 (1968).
8. M. Michaels, *Lancet* **1964-II**.
9. R. O'Meara and M. O'Halloran, *ibid.* **1963-II**; L. Hughes, *ibid.* **1964-II**.
10. V. Laren, B. Mogensen, C. Amris, O. Storm, *Dan. Med. Bull.* **11**, 137 (1964).
11. E. O'Brien, R. Thomas, D. O'Brien, B. Hogan, *Lancet* **1968-I**.
12. G. Klein, *Cancer Res.* **19**, 343 (1959).
13. H. Wexler, *J. Nat. Cancer Inst.* **36**, 641 (1965).

12 August 1968; revised 12 November 1968

Immunologic Enhancement of Tumor Xenografts by Pepsin-Degraded Immunoglobulin

Abstract. *The serums from guinea pigs previously injected with mouse Ehrlich ascites tumor cells were fractionated to obtain γ_2-immunoglobulin. This immunoglobulin was degraded with pepsin to obtain an $F(ab')_2$ fragment. Fresh tumor cells were incubated with immunoglobulin or the fragment and injected into normal guinea pigs. The growth of these cells as tumor xenografts was inhibited by the γ_2-immunoglobulin and enhanced by the $F(ab')_2$ fragment. Similar incubation of tumor cells with normal guinea pig γ_2-globulin or its derived $F(ab')_2$ fragment did not alter subsequent tumor growth.*

Immunoglobulins are classically associated with protective host defense mechanisms. Rabbit immunoglobulin may be cytotoxic for tumor cells in the presence of complement in vitro, but the $F(ab')_2$ fragment prepared by pepsin degradation of the immunoglobulin is noncytotoxic (*1*). The guinea pig $F(ab')_2$ immunoglobulin fragment blocks the cytotoxic activity of intact immunoglobulin (*2*). Also, $F(ab')_2$ immunoglobulin fragments enhance the growth of mouse tumor isografts (*3*) and allografts (*4*). We present evidence that xenografts of mouse Ehrlich ascites tumor cells in guinea pigs are inhibited by γ_2-immunoglobulin, but are enhanced by the $F(ab')_2$ immunoglobulin fragment. We suggest that production of immunoglobulin fragments in vivo could similarly alter a variety of host immune responses.

γ_2-Immunoglobulin was obtained from the serums of guinea pigs previously injected with Ehrlich ascites tumor cells, and the $F(ab')_2$ immunoglobulin fragment was prepared by pepsin degradation (*2*). Normal γ_2-globulin from the serums of normal guinea pigs and its $F(ab')_2$ fragment were obtained in the same manner. The γ_2-immunoglobulin showed a single arc by immunoelectrophoresis when developed with rabbit antiserum to guinea pig serum. In concentrations of 0.06 mg/ml or more, the γ_2-immunoglobulin was cytotoxic for 3×10^6 Ehrlich ascites tumor cells in the presence of guinea pig complement, but was not cytotoxic with heat-inactivated complement. Normal γ_2-globulin and the $F(ab')_2$ fragments from immunoglobulin and normal globulin were not cytotoxic in the presence of complement (*2*).

Seven days after their intraperitoneal implantation into adult male ICR mice, Ehrlich ascites tumor cells were harvested and washed three times with 0.85 percent NaCl solution. Sixty million viable cells, as determined by Safranin O dye exclusion (*2*), were incubated with 1.5 mg of γ_2-globulin or $F(ab')_2$ fragments in the absence of complement in a total volume of 0.5 ml of 0.85 percent NaCl, or with 0.85 percent NaCl alone, for 30 minutes at 37°C. Then 0.4 ml of 0.85 percent NaCl was added, and 20×10^6 cells in 0.3 ml were injected intradermally into the shaved right lower lumbar region of three adult guinea pigs. Tumors were measured along two perpendicular axes, and the arithmetic mean was determined.

Tumors obtained from cells that had been incubated with $F(ab')_2$ immunoglobulin fragments were larger than controls (Fig. 1, A–C). Tumors produced by cells that had been incubated with γ_2-immunoglobulin were smaller than controls. Prior incubation with normal γ_2-globulin or its derived $F(ab')_2$ fragment produced essentially no change in tumor growth (Fig. 1D). Tumors in the $F(ab')_2$ immunoglobulin fragment groups were the last to be rejected (Fig. 1, A and B), although one exceptional tumor in the γ_2-immunoglobulin group showed initial inhibition followed by rapid growth and was not rejected until day 24 (Fig. 1C). In experiment D all tumors were rejected at the same time.

Two inferences can be made from these results. First, during incubation in vitro, γ_2-immunoglobulin combined with tumor cells. After inoculation of the cells, complement of the guinea pig host was fixed by the immuno-

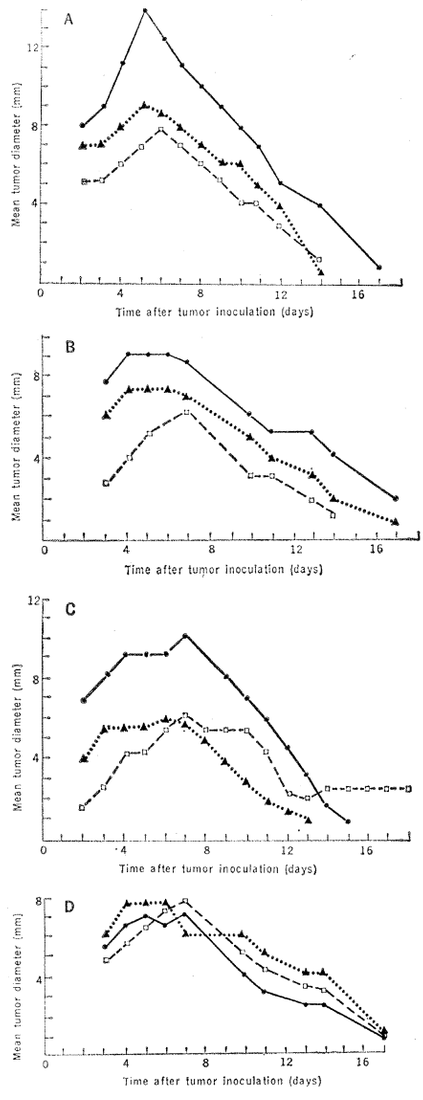

Fig. 1. Immunologic enhancement of mouse tumor xenografts in guinea pigs by pepsin-degraded immunoglobulin. Mouse Ehrlich ascites tumor cells (20×10^6) were incubated with guinea pig γ_2-globulin (□— —□), $F(ab')_2$ globulin fragments (●———●), or 0.85 percent NaCl (▲ · · · ▲) before intradermal inoculation into guinea pigs. Immunoglobulin and its fragment were used in experiments A to C; normal globulin and its fragment in experiment D.