produced. However, the anemia that sometimes occurred could not be related to pyridoxine metabolism.

Despite extensive empirical use of pyridoxine as a therapeutic test agent for many different conditions, its essential role in human hematopoiesis has been demonstrated only in the infant maintained on a synthetic diet lacking pyridoxine. The present observations indicate that abnormal hematopoiesis (associated with abnormalities in the metabolism of tryptophan and iron) can occur in the adult human secondary to a naturally occurring alteration in pyridoxine metabolism. Since the dietary intake of pyridoxine was estimated to be normal, the possibilities exist at present that either an absorptive defect or metabolic aberration accounts for the findings in this patient. The nature of the alteration and its role in the steps involved in hematopoiesis are unknown.

Summary. An adult patient is reported with hematologic abnormalities that were unresponsive to the usual hematopoietic agents, and characterized by a hypochromic anemia, leukocytosis, high serum iron and high per cent iron binding protein saturation. Measurements of the abnormal urinary excretion of the metabolites of l-tryptophan following oral loading tests demonstrated an alteration in the metabolism of this amino acid. The clinical and laboratory abnormalities were promptly reverted to normal, and hematological remission followed intramuscular administration of pyridoxine hydrochloride.

1. Fouts, P. J., Helmer, O. M., Lepkovsky, S., and Jukes, T. H., *J. Nutrition,* 1938, v16, 197.

2. McKibbin, J. M., Schaefer, A. E., Frost, D. V., and Elvehjem, C. A., *J. Biol. Chem.,* 1942, v142, 77.

3. Cartwright, G. E., Wintrobe, M. M., and Humphreys, S., *ibid.,* 1944, v153, 171.

4. Cartwright, G. E., *J. Clin. Invest.,* 1944, v23, 926. (Abstract).

5. ———, *J. Biol. Chem.,* 1948, v172, 557.

6. Poppen, K. J., Greenberg, L. D., and Rinehart, J. F., *Blood,* 1952, v7, 436.

7. Wintrobe, M. M., *Clinical Hematology,* Lea and Febiger, Philadelphia, 1952.

8. Vilter, R. W., *J.A.M.A.,* 1955, v159, 1210.

9. Lepkovsky, S., Roboz, E., and Hargen-Smit, A. J., *J. Biol. Chem.,* 1943, v149, 195.

10. Greenberg, L. D., Bohr, D. F., McGrath, H., and Rinehart, J. F., *Arch. Biochem.,* 1949, v21, 237.

11. Harris, J. W., *J. Lab. Clin. Med.,* in press.

12. Arrowsmith, W. R., Burris, M. B., and Segaloff, A., *ibid.,* 1953, v42, 778.

13. Brown, R. R., and Price, J. M., *J. Biol. Chem.,* in press.

14. Harris, J. W., Price, J. M., and Whittington, R. M., to be published.

15. Snyderman, S. E., Holt, L. E., Jr., Carretero, R., and Jacobs, K., *J. Clin. Nutrition,* 1953, v1, 200.

16. Hunt, A. D., Stokes, J., McCrory, W. W., and Stroud, H. H., *Pediatrics,* 1954, v13, 140.

17. Mueller, J. F., and Vilter, R. W., *J. Clin. Invest.,* 1950, v29, 193.

18. Vilter, R. W., Mueller, J. F., Glazer, H. S., Jarrold, T., Abraham, J., Thompson, C., and Hawkins, V. R., *J. Lab. Clin. Med.,* 1953, v43, 335.

19. Ebaugh, F. G., Jr., Emerson, C. P., and Ross, J. F., *J. Clin. Invest.,* 1953, v32, 1260.

Received January 13, 1956. P.S.E.B.M., 1956, v91.

Acceptance of Tumor Homografts by Mice Injected with Antiserum. II. Effect of Time of Injection.* (22285)

NATHAN KALISS. (With technical assistance of Arthur I. Aronson, Bradley F. Bryant, and Venice M. Bryant.)

Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.

With certain combinations of transplantable tumors and inbred strains of mice, a normally resistant host will accept a tumor homograft if the animal is injected with preparations of mouse tissues prior to tumor grafting(1-4). The altered response of the host may persist for many months after the

* Supported by grant-in-aid from American Cancer Society, upon recommendation of Committee on Growth of National Research Council, and research grant C-1594 from National Cancer Institute, National Institutes of Health, Public Health Service.

tissue has been injected, but tissue administered *after* the tumor is grafted does not induce acceptance of the homograft(5). *Antiserums* to mouse tissues, produced in rabbits or mice, also will ensure survival of a tumor homograft when injected into normally resistant mice(6-9). The present report is a study of the survival of tumor homografts in mice receiving antiserum at different time intervals, with respect to the time of tumor grafting. It was undertaken to establish a comparison with the results previously found for the effects of varying the time of injection of *tissue* preparations(5). This comparison is of importance for an understanding of the mode of action of both the tissue injections and the antiserums. A preliminary report of the present findings has been presented elsewhere(10).

Materials and methods. The transplantable tumor used as the homograft was Sarcoma I (SaI) which is indigenous to the inbred A strain of mice. Hosts were mice of the C57BL/6Ks strain (a C57Black subline), which is genetically unrelated to the A strain. The tumor grows progressively in 100% of strain A mice, and very rarely in an untreated C57BL/6Ks mouse. (A full description of the properties of this tumor has been given previously(11).) All mice were 2 to 4 months old at the start of the experiments. *Antiserums* were produced in rabbits and C57BL/6Ks mice. The rabbits received injections of either freshly secured spleens of strain A mice or freeze-dried SaI tumor. The tissues were prepared as homogenates in sterile 0.85% saline, in an amount of approximately 20 mg/ml dry weight and the homogenates were centrifuged at 4500 x g. The supernatants thus obtained were used for intravenous injection, and the sediments for intraperitoneal injection. The rabbits received a total of 6 injections, spaced 4 days apart. Except for the first injection, in which supernatant was given intravenously, the sediment was injected in the morning, and the supernatant on the afternoon of the same day, to minimize anaphylaxis. The amounts of sediment injected per rabbit were, respectively, 1.0, 1.5, 2.0, 2.0, and 2.0 ml; all injections of supernatant were in the amount of 0.5 ml. The rabbits were bled from the heart while under nembutal anaesthesia. The serums were separated by centrifugation, freeze-dried and stored *in vacuo,* under refrigeration. For use in the experiments, the serums were reconstituted to the original volume with sterile distilled water. They were then heated at 56°C for 1 hour, since it had been found (12) that unheated rabbit antiserum may induce severe shock in mice, with subsequent death, and that the shock is greatly reduced by heating the serums. At the time that they were reconstituted, the serums had been in storage for 2 years and 8 months. In mice, centrifuged supernatants of freeze-dried or frozen SaI tumor were administered in 5 intraperitoneal injections of 0.5 ml each, over a period of 2½ weeks. Each mouse received a total of approximately 7.5 mg dry weight of tissue. Twelve days after the last injection, the mice were bled by incision of the lateral tail vein. An additional bleeding was made 1 month later, after a single "booster" injec-

TABLE I. Effect of Time of Injection of Rabbit Antiserums on Survival of Homografts of a Strain A Tumor in C57BL/6Ks Male Mice.

Interval between inj. of serums and t g*	No. mice dying with tumors; inj. with antiserums from rabbits No.† K3	208
Serum inj. before t g		
28 days	0/ 5	1/5
7 "	0/ 5	4/5
3 to 6 hr	10/10	5/5
	Serum from rabbits No. 2P31	2P26/304‡
3 to 6 hr before	5/ 6	13/13
Serum inj. after t g		
1 day	2/ 6	3/ 5
2		0/ 5
3	0/ 5	
5		0/
7	0/10	

	Normal rabbit serum	Rabbit antiserum to guinea pig spleen	No inj.
4 hr before t g	0/6	0/6	0/27

* t g = tumor grafting.

† Numerators are No. dying with progressively growing tumors; denominators, total No. of animals in each group.

‡ Pooled serum from 2 rabbits.

tion of an equivalent of approximately 4 mg dry weight of tumor supernatant. All the serums were pooled and stored at −26°C. In some of the experiments, the isoantiserums had been produced shortly before they were used; in others they had been in storage for 10 months. The older serums were fully as effective as the fresher ones. Antiserums were administered intraperitoneally to the test animals. The homografts (SaI) were inoculated subcutaneously by trocar, under aseptic conditions, in the suprascapular region. Growth of the grafts was followed by periodic palpation until the mice either died with a progressively growing tumor, or remained without an evident growth for a consecutive period of 2 months, at which time the animals were classified as "negative."

Results. Serums produced in rabbits. (Table I). Four different lots of antiserums were tested, 3 of these from 3 different rabbits, and the fourth a pooled serum from 2 other rabbits. Rabbit K3 (Table I) had been immunized with the strain A tumor, SaI; the other rabbits were immunized with spleen from strain A mice. The serums from rabbits K3 and 208 were administered in 2 injections, per mouse, of 1 ml each, given 24 hours apart. The other 2 serums were administered in a single injection of 1 ml per mouse. The following points are evident from the data: (a) the best results, in terms of homograft survival, were obtained when the antiserum was injected shortly before tumor grafting; and (b) antiserum injected more than one day after tumor grafting did not effect homograft survival. There is a positive correlation between the relative strength of the antiserums from rabbits 208 and K3 and the length of time that their effectiveness remained in evidence when injected prior to tumor grafting. The serum from rabbit 208 exhibited both a higher complement-fixing titer and hemagglutinating titer (with red cells from strain A/Ks mice) than did the serum from rabbit K3. The data of Table I show that the serum from rabbit 208 exhibited some effect at an interval as long as 28 days between its injection and subsequent tumor grafting, while no homografts survived in the mice that received the K3 serum at as short an interval as 7 days before tumor inoculation. However, that there was some effect of the K3 serum given at this time interval was shown by the markedly larger transient growth of the grafts in this group of mice, and their longer persistence before regression, as compared with uninjected controls.

That the results described are due to specific antibody in the passively transferred antiserum is further shown by the lack of effect of normal rabbit serum, or rabbit antiserum to guinea pig spleen, and by data from a previous study on serum fractions(8).

Serums produced in C57BL 6Ks Mice (Table II). The isoantiserum was adminis-

TABLE II. Effect of Time of Injection of Isoantiserum, produced in C57BL/6Ks Mice, on Survival of Homografts of a Strain A Tumor in C57BL/6Ks Mice.

Interval between inj. of serum and t g*	No. mice dying with tumors ♀	♂
Serum inj. before t g		
9 wk	0/ 5	0/ 5
5	0/ 5	3/ 5
1	5/ 5	5/ 5
4 to 6 hr†	14/15	20/21
Serum inj. after t g		
1 day	—	5/ 5
3	—	5/ 5
7	—	4/ 5
Normal C57BL/6Ks serum, 4 to 6 hr before†	0/ 5	0/17
Nothing inj.	0/ 5	0/10

* t g = tumor grafting.
† Data of 4 experiments.

tered in a single injection of 1 ml per mouse. As with the antiserums produced in rabbits (Table I), the effect of the isoantiserums was more marked as the time interval of their injection, prior to tumor grafting, was decreased. The effectiveness of the isoantiserum, however, remained in evidence for a longer time interval than did the rabbit antiserum. With the isoantiserum, there was a marked transient growth of the grafts (as compared with untreated controls) in the mice injected as long as 9 weeks prior to tumor grafting. This was particularly evident in the male hosts, in whom palpable

grafts were still present 18 days after tumor inoculation, while in the untreated controls the grafts had completely regressed by 13 days.

In the group receiving isoantiserum 5 weeks before tumor grafting, there was a marked transient growth of the tumors in all 5 males. In 3 of the 5 males, the grafts resumed progressive growth after a slight regression, with eventual death of the mice. In the other 2 males, eventually classified as "negative," tumor nodules were palpable for 25 days after grafting. The grafts in the 5 females in this group also showed a large growth before regression, as compared with untreated controls, though they did not reach as large a size as in the males.

The difference between the sexes in the rate of growth of the grafts was also evident in the groups receiving isoantiserum 1 week before, or on the same day as, tumor inoculation. There consistently was a more rapid growth of the grafts in the males. Another difference between the effect of the isoantiserum and the rabbit antiserums was the survival of homografts in the mice receiving isoantiserum as long as 7 days *after* tumor inoculation. No homografts had survived in the mice receiving rabbit antiserum more than 1 day after tumor grafting.

Effect of dosage of antiserum and time of administration. The assumption was tested that dosage of antiserum might account for the differences observed between the effectiveness of rabbit antiserum and isoantiserum administered *after* tumor inoculation. This assumption was based on two premises: that more antibody is needed to insure the survival of a homograft after the immune responses of the host had been initiated; and that more antibody of the type ensuring homograft survival would be found, per unit volume, in isoantiserum than in the rabbit antiserum. Two groups of experiments were done, one with the gamma-globulin fraction of rabbit antiserum, and the other with whole isoantiserum produced in C57BL/6Ks mice. The gamma-globulin was used because it was known that it is active in inducing homograft survival (8). Furthermore, it was felt that the mice would tolerate better larger doses of the gamma-globulin than of whole serum, since it had been found that whole rabbit antiserum, even though heated to 56°C, will induce some degree of shock, and that the gamma-globulin fraction does not produce this reaction(12). Gamma-globulin was prepared by salt fractionation of an antiserum to spleen from strain A/Ks mice. It was then freeze-dried and reconstituted to the desired concentrations, on a dry weight basis, with sterile 0.85% NaCl.

The data for 2 separate experiments with gamma-globulin are given in Table III. In the groups receiving globulin at 4 or 7 days after tumor grafting, there is a positive correlation between the amount of globulin administered and the number of mice dying with progressively growing tumors. A marked growth of the grafts, which was initiated several days after the injection of globulin, was observed in almost all the mice in these same groups, whether or not the grafts were destined to grow progressively. In many of the grafts that eventually grew progressively, to death of the host, this spurt in growth was followed by a progressive diminution in size and then a resumption of growth.

TABLE III. Effect of Dosage and Time of Injection of Rabbit Antiserum Gamma-Globulin on Survival of Homografts of a Strain A Tumor in C57BL/6Ks Male Mice.*

Time γ-globulin inj. after t g†	No. mice dying with tumors at γ-globulin dosages/mouse of			
	10 mg (Exp. 1)	5 mg (Exp.1)	1 mg Exp. 1	1 mg Exp. 2
Same day	—	—	5/5	5/5
1 day	5/5	5/5	5/5	—
3	—	—	—	0/5
4	3/4	2/5	1/5	—
5	—	—	—	0/5
7	2/5	0/5	1/5	—
Nothing inj.		1/5 (Exp. 1)		
3, 5, 7, 9 days after t g‡		0/5 (Exp. 2)		

* Gamma-globulin obtained by fractionation with $(NH_4)_2SO_4$.
† t g = tumor grafting.
‡ 4 inj./mouse; 1 mg/inj.

The experiment with the isoantiserum produced in C57BL/6Ks mice was carried out at 3 different dosage levels of serum, the amount

administered per mouse being given as a single injection of either 1.0 ml, 0.5 ml, or 0.25 ml. The data are presented in Table IV.

TABLE IV. Effect of Dosage and Time of Injection of Isoantiserum, Produced in C57BL/6Ks Mice, on Survival of Homografts of a Strain A Tumor in C57BL/6Ks Male Mice.

Time serum inj. after tumor grafting	No. mice dying with tumors, at serum dosages/mouse of		
	1 ml	0.5 ml	0.25 ml
2 days	5/5	5/5	5/5
4	4/5	3/5	2/5
7	3/5	3/5	1/5
10	1/5	3/5	2/5
14	0/5	0/5	0/5

There is no clear-cut evidence of differences due to dosage. In the groups of mice that died with tumors, and that had received antiserum 4 days, or longer, after tumor inoculation, there characteristically was an initial growth followed by a diminution in the size of the grafts, even for some time after the mice had received the antiserum, followed by a gradual resumption of growth. This experiment demonstrates, incidentally, that the grafts may remain viable in the foreign host for at least as long as 10 days after inoculation, a finding which is in agreement with previously published reports(5,13).

Discussion. The time relationships obtained with the antiserums are in contrast with those obtained in mice injected with tissue extracts(5). Homografts survived in mice inoculated with a tumor graft as long as 11 months after the mice had received an injection of tissue. On the other hand, tissue injected more than one day *after* tumor grafting did not induce homograft survival. With the *antiserums,* there was a diminution of their effectiveness as the interval of time between the administration of the serum and the subsequent grafting of the tumor was increased. This undoubtedly is attributable to the continuous elimination of the serum by the host. The injection of antiserum as long as 4, 7, or 10 days *after* tumor grafting ensured survival of a significant proportion of the homografts.

These observations, together with data obtained with cortisone-treated mice(9,14), indicate that the production of antiserum in mice pretreated with tissues may be a necessary condition for the survival of the tumor homografts. (Though the data show that the effects of the antiserums produced in rabbits and mice are the same, the possibility that there are qualitative differences in their mode of action is not ruled out.)

Three alternative hypotheses may be considered as to the mode of action of the antiserum. (a) The antiserum "coats" the specific tumor antigens which normally would evoke a "homograft reaction" by the host. Such coated antigens presumably would not stimulate the tissues (lymph nodes(13), and possibly other tissues) which otherwise would react to destroy the graft. (b) The antiserum acts directly on the reactive centers (lymph nodes, etc.) of the host to block the "homograft reaction," whether or not the animal had been previously exposed to the immunizing stimulus of a tumor inoculum. (c) The antiserum acts directly on the graft, inducing an adaptive alteration which permits the graft to survive in an otherwise hostile environment.

Our findings make hypothesis (a) untenable, since antiserum injected at 4, 7, or 10 days *after* tumor inoculation, led to survival of the grafts. That these time intervals should be sufficient to develop the immune responses of the host has been shown by Mitchison(13), who demonstrated that immunity to a tumor homograft could be passively transferred by lymph nodes taken from mice 3 to 10 days after the donors had been exposed to the immunizing stimulus of a homograft inoculum.

Whether hypothesis (b) is valid cannot be established from the data of the present paper. However, from data of other experiments it is believed that this hypothesis is open to question. A fuller consideration of this point has been given elsewhere(14).

The possibility that hypothesis (c) is reasonable is supported by the reports of Barrett and Deringer(15,16), and our own data (12), that a tumor homograft that survives in mice of a "foreign" strain loses some of its transplantation specificity, since it will grow

progressively when grafted to untreated mice of inbred strains in which it normally would be rejected. Barrett and Deringer used a transplantable tumor, indigenous to the C3H strain, that had been grown in F_1 (C3H x C) mice. Though the tumor grew in 100% of the F_1 mice, these hosts can be considered as "foreign" to the tumor to the extent that half of the animals' heredity came from strain C parentage. When grafts derived from the "F_1" hosts were implanted into backcross (F_1 x C3H) mice, three times as many mice died with progressively growing tumors, as compared with backcross mice receiving their grafts directly from C3H mice. Barrett and Deringer(15) concluded that " . . . for the moment it is best to describe (the change in the "F_1" tumors) more loosely as an induced adaptation in the tumor." In our own case, the altered grafts came from a strain of mice in which survival of the tumors had been induced by pretreating the hosts with freeze-dried tumor tissue. On subsequent transplantation, the tumors grew progressively in untreated mice of an inbred strain which normally rejects the tumor. However, for reasons unknown, this change in graft specificity was lost after 7 transplant generations. An accelerated tumor growth in mice pretreated with killed tumor tissue, even in animals of the strain to which the tumor is indigenous, has been reported by Casey, Laster, and Ross (17). This characteristic was retained in subsequent transplant generations. Whether these examples are pertinent to our case would depend both upon the demonstration of the production of antiserum under the experimental conditions used by these investigators, and the effects of such an antiserum, if it is produced, upon the growth characteristics of the tumor grafts.

Another point brought out by our data is that the effects of the antiserums injected prior to tumor grafting are indicated not only by those cases where the grafts grew progressively, with eventual death of the host, but also by the numerous instances in which there was a marked transient growth of the grafts before they regressed. The latter cases are most probably an expression of submimal doses of antiserum present in the host at the time of tumor inoculation. It may be assumed that the number of tumor cells affected by the antiserum was insufficient to ensure continued progressive growth of the grafts.

In conclusion, it is emphasized that a definitive answer as to the mode of action of the antiserum must await further experimentation, and that the mechanisms involved may be concerned with processes as yet unknown, and perhaps other than those that have been considered in this paper.

Summary. Passively transferred antiserum to mouse tissues, produced in rabbits or mice, will induce the survival of a tumor homograft in mice. The best effect, in terms of homograft survival, is obtained if the antiserum is administered to the prospective host shortly before, or shortly after, tumor grafting. The possible mode of action of the antiserum in ensuring homograft survival is considered.

1. Snell, G. D., Cloudman, A. M., and Woodworth, E., *Cancer Res.*, 1948, v8, 429.

2. Kaliss, N., and Snell, G. D., *ibid.*, 1951, v11, 122.

3. Snell, G. D., *J. Nat. Cancer Inst.*, 1952, v13, 719.

4. Day, E. D., Kaliss, N., Aronson, A. I., Bryant, B. F., Friendly, D., Gabrielson, F. C., and Smith, P. M., *ibid.*, 1954, v15, 145.

5. Kaliss, N., and Day, E. D., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 115.

6. Kaliss, N., and Molomut, N., *Cancer Res.*, 1952, v12, 110.

7. Kaliss, N., Molomut, N., Harriss, J. L., and Gault, S. D., *J. Nat. Cancer Inst.*, 1953, v13, 847.

8. Kaliss, N., and Kandutsch, A. A., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 118.

9. Kaliss, N., *Annals N. Y. Acad. Sci.*, 1955, v59, 385.

10. Kaliss, N., *Transpl. Bull.*, 1955, v2, 8.

11. Kaliss, N., Borges, P. R. F., and Day, E. D., *Cancer Res.*, 1954, v14, 210.

12. Kaliss, N., unpublished data.

13. Mitchison, N. A., *J. Exp. Med.*, 1955, v102, 157.

14. Kaliss, N., *Annals N. Y. Acad. Sci.*, in press.

15. Barrett, M. K., and Deringer, M. K., *J. Nat. Cancer Inst.*, 1950, v11, 51.

16. ———, *ibid.*, 1952, v12, 1011.

17. Casey, A. E., Laster, W. R., and Ross, G. L., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 358.

Received January 27, 1956. P.S.E.B.M., 1956, v91.