7. V. C. Dewey and G. W. Kidder, *J. Chromatogr.* **31**, 326 (1967).
8. H. Rembold and H. Metzger, *Chem. Ber.* **96**, 1395 (1963).
9. E. M. Gál, R. D. Heater, S. A. Millard, *Proc. Soc. Exp. Biol. Med.* **128**, 412 (1968).
10. G. Guroff, C. A. Rhoads, A. Abramowitz, *Anal. Biochem.* **21**, 273 (1967).
11. M. Matsubara, S. Kato, M. Akino, S. Kaufman, *Biochim. Biophys. Acta* **122**, 202 (1966).
12. E. M. Gál, A. E. Roggeveen, S. A. Millard, *J. Neurochem.* **17**, 1221 (1970).
13. D. B. Fisher and S. Kaufman, *J. Biol. Chem.* **247**, 2250 (1971).
14. We thank S. A. Millard of our laboratory for performing the assays with the *Pseudomonas* enzyme, and G. Guroff of the National Institutes of Health, Bethesda, Md., for this enzyme.
* Present address: Division of Physiological Sciences, School of Veterinary Medicine, University of California, Davis 95616.

11 October 1972

Graft versus Leukemia: Quantification of Adoptive Immunotherapy in Murine Leukemia

Abstract. *Quantification of the antileukemic reactivity of transplanted immunocompetent cells from various allogeneic donors was achieved against a long-passage lymphocytic leukemia of AKR mice. Adoptive immunotherapy was the exclusive antileukemic treatment. Cells from DBA/2 donors exhibited maximal antileukemic effect, inactivating up to an estimated 10^7 leukemia cells. The cellular events were interpreted by using a theoretical cytokinetic construct.*

Immunotherapy generally is acknowledged to be most effective against what Woodruff has called "the minimal residual cancer" (*1*). Much experimental data have demonstrated that leukemia cells can be killed by adoptive immunotherapy (*2*). In the experiments reported here, we have attempted to quantify the number of leukemia cells killed (or inactivated) by a graft-versus-leukemia (GVL) reaction (*3*).

The experimental model was designed to permit an estimate of the number of leukemia cells killed, and so the measured antileukemic effect would be the exclusive result of the GVL reaction. The immunological defense system of the AKR (*H-2*^{k}) host mice was severely damaged by total body x-irradiation of 800 r, a supralethal dose (100 percent of animals dead within 15 days) in this strain. The irradiation conditions have been described (*4*). Irradiation was given the morning of day 0 as immunosuppression in order to (i) impair or eliminate host immunological participation against the leukemia and (ii) prevent rejection of the immunocompetent cells to be transplanted the following day. There was no antileukemic effect from the irradiation because it was administered before the leukemia cells were inoculated. Doses of 1, 4, 16, or 64 $\times$ 10^3 "blast" (large) cells from the spleen of an AKR mouse bearing BW5147, a long-passage lymphocytic leukemia (*5*), were given intravenously to the irradiated AKR primary hosts on the afternoon of day 0. Blast cells comprised 52 to 77 percent of cells in the spleen cell suspensions. After a delay of 24 hours, in which the leukemia cells were permitted to establish residence and resume multiplication, the mice were given separate intravenous injections of 2 $\times$ 10^7 bone marrow cells and 10^7 lymph node cells from one of four allogeneic donor strains: CBA (*H-2*^{k}), identical with the recipient at the *H-2* locus; and A (*H-2*^{a}), C57BL/6 (*H-2*^{b}), and DBA/2 (*H-2*^{d}), mismatched at this locus. The methods for preparation of the cell suspensions have been reported (*4*). The transplant on day 1 of allogeneic immunocompetent cells to the immunosuppressed, leukemic AKR mice (adoptive immunotherapy or GVL reaction) was the only therapy employed. The GVL reaction was allowed to proceed for 6 days, a period shown to be the minimal effective duration (*6*). After the 6-day GVL reaction, the primary hosts were killed and their spleens were removed. All spleen cells obtained from each primary host were injected intraperitoneally into one normal AKR secondary recipient. Secondary recipients were observed for survival. Those that died were autopsied, and all were found to have leukemia. None of the secondary recipients died after day 46, and an end point of 60 days was selected for analysis of the data.

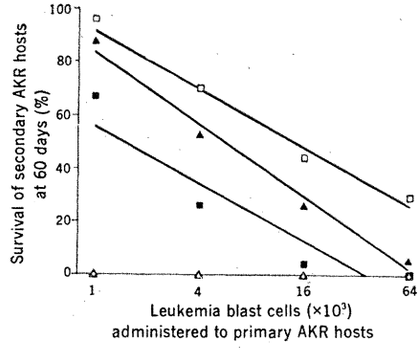


Fig. 1. Dose-response curves for survival of AKR secondary recipients at 60 days after spleen transfers from immunosuppressed AKR mice. The immunosuppressed mice had received graded doses of leukemia cells and had been treated with immunocompetent cells from allogeneic donors: □, DBA/2; ▲, C57BL/6; ■, A; △, CBA; and ○, no cells.

On the basis of three observations we elected to use the spleen as the organ for bioassay. First, Skipper *et al.* (*7*) reported that long-passage AKR leukemia invariably involved the spleen. Second, histologic examination in our laboratory of the tissues of more than 500 control AKR mice that died of BW5147 long-passage leukemia has invariably shown massive splenic involvement. Third, bioassay studies by Valeriote *et al.* (*8*) of a variety of tissues in AKR mice bearing a long-passage lymphocytic leukemia showed that the spleen contained one or more clonogenic leukemia cells after doses of cyclophosphamide that usually killed all detectable malignant cells in other organs. These results suggested that spleen cell transfer for the bioassay would afford us the greatest probability for recovery of residual leukemia cells, and that "cell cure" of the spleen might well connote "cell cure" of the entire animal.

The experimental results are presented in Table 1 and Fig. 1. The data provide evidence that immunocompetent cells from unprimed donors were capable of inactivating or killing leukemia cells and that this effect was accomplished in 6 days. Also apparent is the relative effectiveness of immunocompetent cells from the various donor strains, and the dose-response relation for each donor strain.

All secondary recipients died of leukemia after the transfer of spleen cells from control mice that were not treated with adoptive immunotherapy (Table 1). Similarly, all secondary recipients died of leukemia after spleen cell transfer from leukemic AKR mice that had been treated with immunocompetent cells from allogeneic donors that were identical at the *H-2* locus (CBA donors in Table 1). In other words, cells from CBA donors were ineffective at eliminating leukemia from AKR mice that had received as few as 10^3 BW5147 leukemia cells 1 day earlier.

Definite antileukemic activity was exhibited by immunocompetent cells

Table 1. Effect of adoptive immunotherapy on immunosuppressed AKR ($H\text{-}2^k$) mice that have been given graded doses of leukemia blast cells. Shown are percentages of survival of secondary recipients at 60 days; *N*, number of secondary recipients tested.

Donor strain for GVL reaction	Survival of bioassay mice: number of leukemia blast cells administered to primary AKR hosts							
	1,000		4,000		16,000		64,000	
	Percent	*N*	Percent	*N*	Percent	*N*	Percent	*N*
DBA/2 ($H\text{-}2^d$)	96	24	71	24	44	23	29	24
C57BL/6 ($H\text{-}2^b$)	88	32	53	32	26	31	6	31
A ($H\text{-}2^a$)	67	24	26	23	4	24	0	24
CBA ($H\text{-}2^k$)	0	23	0	24	0	32	0	30
None (controls)	0	38	0	38	0	41	0	42

derived from allogeneic donors that differed from AKR mice at the *H-2* locus (Fig. 2 and Table 1). The three slopes in Fig. 2 showed no significant difference when tested for parallelism, and were thus considered to be parallel. Parallelism in these dose-response curves suggests that the mechanism for antileukemic effect was similar for the immunocompetent cells from DBA/2, C57BL/6, and A donors (*9*). Covariance analysis of antileukemic potency disclosed a significant difference ($P < .05$) between immunocompetent cells from DBA/2, C57BL/6, and A donors. In the dose range tested, immunocompetent cells from all *H-2* incompatible donors exhibited greater antileukemic activity than did cells from *H-2* compatible CBA donors ($P < .01$).

A theoretical cytokinetic construct was prepared in order to estimate the number of leukemia cells killed by the GVL reaction (Fig. 2). After the inoculation of 10^3 BW5147 blast cells on day 0 (*a*, Fig. 2), their growth in AKR primary recipients was as shown (*b*, Fig. 2). Without treatment the median survival time of animals was 14.1 days. The generation time of cells was found to be approximately 16 hours (*10*) until a total of about 10^8 leukemia cells were present. As suggested by Skipper *et al.* (*11*), the growth rate was then presumed to slow slightly until death, when about 10^9 leukemia cells were estimated to be present in the animals.

We have assumed, as did Bruce *et al.* (*12*), that the growth of leukemia cells in the spleen paralleled that in the rest of the body (*c*, Fig. 2). Immunocompetent cells were administered on day 1 (*d*, Fig. 2). If one assumes no perturbation of the leukemia cell cycle, most of the leukemia cells had divided at least once by day 1, and the mice that originally received 10^3 leukemia cells held an estimated 2.7×10^3 leukemia cells. We showed previously that transplanted immunocompetent cells were incapable of mounting an effective immunotherapeutic attack against this leukemia in 4 days (*6*). One would anticipate a time delay between transplantation of the immunocompetent cells and a significant antileukemic effect. Time would be necessary to enable the transplanted immunocompetent cells to establish residence in the foreign host, undergo antigen recognition, and proceed through the steps necessary to carry out the desired immunologic attack against the leukemia cells. Therefore, we estimated a delay of approximately 4 days before the GVL reaction started killing leukemia cells (*e*, Fig. 2). We have assumed that the generation time of the leukemia was unchanged during this 4-day delay. Therefore, on day 5, when the GVL reaction presumably became effective, the AKR primary hosts that originally received 10^3 leukemia cells held an estimated 1.5×10^5 leukemia cells. At this same time, the spleens of these mice held an estimated 7.5×10^4 leukemia cells.

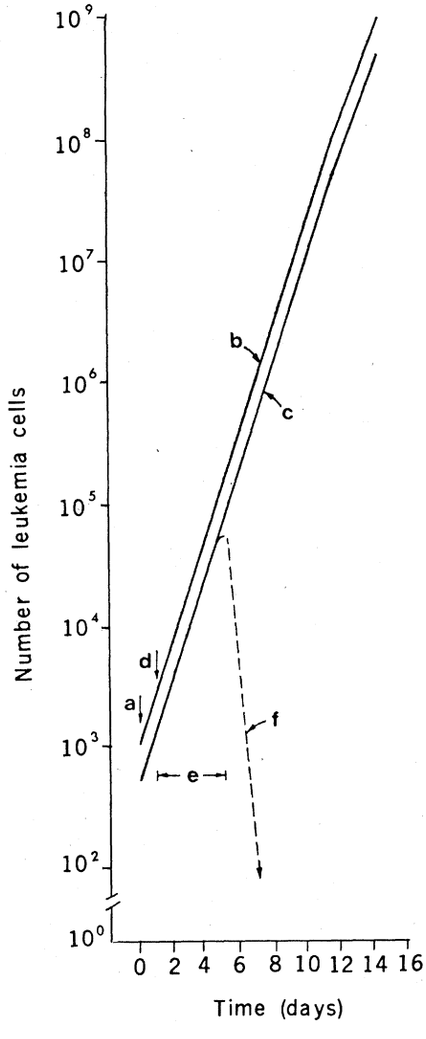


Fig. 2. Cytokinetic diagram to explain cellular events of experimental model. Symbols are *a*, inoculation of leukemia cells into primary recipient; *b*, growth of cells in primary recipient; *c*, growth of cells in spleen; *d*, administration of immunocompetent cells; *e*, delay before GVL reaction started to kill leukemia cells; and *f*, effect of GVL reaction on leukemia cell survival. A complete description is in the text.

We postulate an abrupt wave of injury with rapid kill of leukemia cells by immunocompetent cells from donors that were mismatched at the *H-2* locus. In previous studies (*10*), we found that approximately 1 percent of the BW5147 leukemia cells are clonogenic. Let us assume that the clonogenic cells in the spleen cell suspensions followed a Poisson distribution and, further, that there were 10^2 leukemia cells remaining in each spleen after therapy. Then about 63 percent of the secondary recipients would be expected to receive at least one clonogenic leukemia cell and would develop leukemia. Because less than 63 percent of the secondary recipients developed leukemia, it follows that the number of leukemia cells remaining in the spleens of the primary hosts 6 days after the initiation of the GVL reaction was less than 10^2 (*f*, Fig. 2).

If one applies this same reasoning to those experiments in which the primary hosts received 64×10^3 leukemia cells on day 0, then the mice held an estimated 10^7 leukemia cells (with approximately 5×10^6 leukemia cells in their spleens) when the GVL reaction became effective. Inasmuch as immunocompetent cells from DBA/2 donors killed sufficient leukemia cells in primary hosts that had received 64×10^3 leukemia cells to permit 29 percent

of the secondary recipients to survive (Table 1), it is possible to estimate that the GVL reaction, as employed in these experiments, reduced the number of leukemia cells from about 10^7 to close to 10^2.

Because of the many assumptions made in attempting to analyze the cellular events which took place in these experiments, it is not possible to state the precise number of leukemia cells killed by the GVL reaction. Nonetheless, some of the capacities and limitations of adoptive immunotherapy are pointed up when the data are reviewed within the framework of current cytokinetic theory. The results reported here suggest that adoptive immunotherapy, under carefully controlled conditions, may prove useful as an adjunct to conventional antileukemic therapy.

MORTIMER M. BORTIN
ALFRED A. RIMM
EDWARD C. SALTZSTEIN
May and Sigmund Winter Research Laboratory, Mount Sinai Medical Center, Milwaukee, Wisconsin 53233, and *Department of Medicine, Biostatistics, and Surgery, Medical College of Wisconsin, Milwaukee*

References and Notes

1. M. F. A. Woodruff, *Brit. Med. J.* **2**, 486 (1970).
2. Two examples of the efficacy of adoptive immunotherapy were reported by G. Mathé, J. L. Amiel, L. Schwarzenberg, A. Cattan, and M. Schneider [*Cancer Res.* **25**, 1525 (1965)] and by M. Boranic [*J. Nat. Cancer Inst.* **41**, 421 (1968)].
3. We employ "GVL reaction" to signify the adoptive immunotherapeutic effect of transplanted immunocompetent cells against leukemia cells in an immunosuppressed host.
4. M. M. Bortin and E. C. Saltzstein, *Science* **164**, 316 (1969).
5. We have found that the administration of 10^5 BW5147 blast cells resulted in steep and highly reproducible mortality patterns despite the use of spleen cell suspensions in which blast cells comprised 4 to 92 percent of cells. Conversely, mortality patterns were not uniform when fixed numbers of spleen cells were given with variable numbers of blast cells.
6. E. C. Saltzstein, J. S. Glasspiegel, A. A. Rimm, R. H. Giller, M. M. Bortin, *Cancer Res.* **32**, 1658 (1972).
7. H. E. Skipper, F. M. Schabel, Jr., M. W. Trader, W. R. Laster, Jr., *Cancer Chemother. Rep.* **53**, 345 (1969).
8. F. A. Valeriote, W. R. Bruce, B. E. Meeker, *J. Nat. Cancer Inst.* **40**, 935 (1968).
9. D. Finney, *Statistical Method in Biological Assay* (Griffin, London, ed. 2, 1964), p. 108.
10. A. A. Rimm, M. M. Bortin, E. C. Saltzstein, *Fed. Proc.* **31**, 768 (1972).
11. H. E Skipper, F. M. Schabel, Jr., M. W. Trader, W. R. Laster, Jr., L. Simpson-Herren, H. H. Lloyd, *Cancer Chemother. Rep.* **56**, 273 (1972).
12. W. R. Bruce, F. A. Valeriote, B. E. Meeker, *J. Nat. Cancer Inst.* **39**, 257 (1967).
13. Supported by grants from the Board of Trustees, Mount Sinai Medical Center, from the American Cancer Society (grant ET-55), and from a memorial to William Heller, Sr. We thank E. Reynolds, M. Hilger, and C. Yuan for expert technical assistance.

20 November 1972

Juvenile Hormone Mimics: Effect on Cirriped Crustacean Metamorphosis

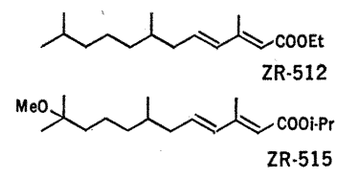
Abstract. *A synthetic juvenile hormone mimic has been shown to cause premature metamorphosis of the cyprid larva of an acorn barnacle in concentrations as low as 10 parts per billion in filtered seawater. The effect of a juvenile hormone mimic on a crustacean has not previously been demonstrated.*

In 1956 Williams (*1*) recognized the potentialities of the cecropia juvenile hormone as an insecticide. Shortly after the structure of the juvenile hormone became known (*2*), and synthetic material had been tested as an insecticide, it became apparent that the cecropia juvenile hormone lacked the necessary chemical stability and species specificity for widespread agricultural use. Meanwhile, juvenile hormone activity was demonstrated for several natural and synthetic compounds. Moreover, some of these compounds exhibited the desired chemical stability and some specificity (*3*).

Two reports led us to believe that juvenile hormone mimics might affect the metamorphosis of crustaceans. Schneiderman and Gilbert (*4*) found that the richest source of juvenile hormone outside of insects was the eyestalk of Crustacea and suggested that juvenile hormone plays a role in crustacean physiology. Furthermore, closely related steroidal molting hormones have been isolated from both insects and crustaceans (*5*).

For the test organism we decided on the acorn barnacle, *Balanus galeatus* (L.) because one of us (E.D.G.) was thoroughly familiar with its rearing and development from larval to adult stages (*6*). The barnacles, members of the subclass Cirripedia, possess a developmental pattern that is analogous to that of the holometabolous insects. Acorn barnacles generally pass through six free-swimming, feeding larval stages (nauplii), followed by a distinct motile but nonfeeding cyprid stage whose function is to find a suitable substrate for settlement where it will attach and metamorphose into the sessile adult form.

We chose to test the juvenile hormone mimics ZR-512 (ethyl 3,7,11-trimethyldodeca-2,4-dienoate) and ZR-515 (isopropyl 11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate)



since both compounds were undergoing field trials as insecticides. The two insect hormone mimics were dissolved in ethanol to make stock solutions (25 mg/ml) which were stored at − 10°C. Aliquots were diluted such that addition of 0.5-ml portions of the ethanolic solutions to 200 ml of filtered seawater yielded final concentrations ranging from 0.1 to 500 ppb (parts per billion). Ethanol (0.5 ml) was added to the filtered seawater (200 ml) in the control experiments.

Adult *B. galeatus* collected from the nearshore waters off the coast at La Jolla provided the larvae, which were reared through naupliar and cyprid larval stages according to established methods (*6*). Preliminary attempts to test the juvenile hormone mimics on the naupliar stages proved unsuccessful and were abandoned. Tentative results showed no apparent effects on nauplii through stage 4, although it was determined that hormone mimic concentrations greater than 500 ppb were lethal to larvae.

Cyprids which were kept at room temperature (25° ± 2°C) in beakers containing various concentrations of ZR-512 in seawater metamorphosed prematurely without attaching to a substrate (*7*). While cyprids normally continue to swim or "walk" at least 30

Table 1. Terminal state of cyprids in ZR-512 experiments. The average duration of each experiment was 6 days. The total number of cyprids was 1129; M, metamorphosed; U, unmetamorphosed; *N*, number of replicate experiments.

ZR-512 (ppb)	Number of cyprids			*N*
	M	U	Dead	
Control	0	131	27	6
0.1	0	71	8	4
1	0	92	12	5
10	140	14	29	9
50	158	0	11	6
100	141	0	17	7
250	76	0	6	4
500	180	2	14	8