Biochemical Pharmacology, Vol. 27, pp. 2507–2511.

0006-2952/78/1101-2507$02.00/0

MECHANISM OF ENHANCED TOXICITY OF 6-MERCAPTOPURINE WITH ENDOTOXIN*

TOMIHIKO HIGUCHI, TORU NAKAMURA and HARUTO UCHINO

Department of Internal Medicine, Shiga Center for Adult Diseases, 328 Moriyama, Moriyama, Shiga, 524 Japan, and The First Division, Department of Internal Medicine, Faculty of Medicine, Kyoto University, Kyoto, 606 Japan

(*Received* 12 *September* 1977; *accepted* 14 *December* 1977)

Abstract—The enhanced toxicity of 6-mercaptopurine with endotoxin has been confirmed with BALB/c male mice. Striking increases in thioinosinic acid were seen in mice pretreated with endotoxin before injection of 6-mercaptopurine, and were in agreement with the time course of the inhibiting effect of endotoxin on sleeping time. However, there were no significant differences in hypoxanthine-guanine phosphoribosyltransferase between control mice and endotoxin-treated mice. Furthermore, there were significant increases in xanthine oxidase after treatment with endotoxin. It is clear that metabolism of 6-mercaptopurine might be modified by activity of the drug-metabolizing enzyme in mouse liver. Based on the effect of endoxotin on 6-mercaptopurine metabolism and the enzymes responsible for 6-mercaptopurine, it is suggested that death caused by 6-mercaptopurine–endotoxin combinations is due to an enhancement of the lethal effect of 6-mercaptopurine. Simultaneous administration of 6-mercaptopurine and endotoxin resulted in a parallel potentiation of either 6-mercaptopurine toxicity or endotoxin toxicity. This interaction was abolished in mice made resistant to the effects of endotoxin. Based on time of death, it is suggested that death caused by 6-mercaptopurine–endotoxin combinations is due to an enhancement of the lethal effect of endotoxin. These results indicate that the underlying mechanism of the interaction may involve a dualistic enhancement of each agent.

6-Mercaptopurine (6-MP) must be converted to thioinosinic acid (TIMP) to be biologically active. This conversion is catalyzed by hypoxanthine-guanine phosphoribosyltransferase (HGPRTase). Thiouric acid is the principal catabolic product of 6-MP and is itself inactive as an antileukemic agent. The inactivation of 6-MP to thiouric acid is catalyzed by xanthine oxidase [1].

A large number of publications in recent years have been based upon the increasing importance of Gram-negative bacillary infections in patients with leukemia [2, 3]. A recent study has also demonstrated enhancement of toxicity of 6-MP, a commonly used antileukemic agent in man [4], by endotoxin. At present, the role of endotoxin in this enhanced toxicity has not been determined. It has been discovered that endotoxin alters hepatic microsomal enzyme activity [5]. Yoshimura [6] recently reported that 6-MP can be desulfurated *in vitro* into a biologically inactive substance by hepatic microsomal enzymes from rats. Most of the endotoxin administered is rapidly removed from the circulation by macrophages of hepatic and splenic sinusoids, and a prominent feature of the toxicity of 6-MP in mice is hepatic damage [7, 8].

The purpose of the present study has been to investigate in detail the effect of endotoxin on the metabolism of 6-MP in mouse liver and on enzymes involved in 6-MP metabolism, in order to clarify the mechanism of the enhanced toxicity of 6-MP with endotoxin. We have also investigated the effects of inorganic phosphate and inosine on the enhanced toxicity of 6-MP with endotoxin.

MATERIALS AND METHODS

Phosphoribosyl-pyrophosphate (PRPP), purine bases, nucleosides and nucleotides were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. [8-^{14}C]6-MP (2.5 mCi/m-mole) and [8-^{14}C]hypoxanthine (20.7 mCi/m-mole) were purchased from the Radiochemical Center, Amersham, England. 6-MP was purchased from Kojin Co., Ltd., Tokyo, Japan. Endotoxin was purchased as a single lot of lipopolysaccharide from *Escherichia coli* from Difco Laboratory, Detroit, MI, U.S.A.

BALB/c male mice weighing 25–29 g were used in all experiments. Mice were obtained from Nippon Rat Co, Saitama, Japan. They were housed in individual cages in air-conditioned rooms where they were illuminated daily from 9.00 a.m. to 5.00 p.m., and were raised on a pellet diet (CE-2: CLEA Japan Inc., Tokyo, Japan). Endotoxin, dibasic disodium phosphate anhydrous and inosine were dissolved in 0.9% NaCl, and 6-MP was dissolved in 0.1 N NaOH, so that 0.01 ml/g of mouse weight would provide the desired dose when administered. All mice were injected intraperitoneally between 9 and 10 a.m. Control mice were given an equal volume of 0.9% NaCl or 0.1 NaOH and then fasted because treated mice could not eat anything. For each assay, the mice were killed and livers were rapidly removed,

* This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education and the Ministry of Health and Welfare of Japan.

B.P. 27/21 c

Table 1. Effect on mortality of varying relative times of administration of 6-mercaptopurine and endotoxin

	Survivors at 3 days					
	Time of administration of endotoxin					
	−24 hr	−16 hr	−6 hr	−0 hr	+6 hr	+24 hr
6-MP (100 mg/kg) at 0 hr + endotoxin (4 mg/kg)	10/10	10/10*	2/10	0/10	0/10	10/10*
6-MP (100 mg/kg) at 0 hr + endotoxin (1 mg/kg)			10/10*	3/10	3/10	
6-MP (75 mg/kg) at 0 hr + endotoxin (2 mg/kg)			2/8	2/8	6/11	
6-MP (50 mg/kg) at 0 hr + endotoxin (4 mg/kg)			6/10	3/6	7/8†	

* Significantly different from mice treated with endotoxin and 6-MP simultaneously ($P < 0.01$).
† Significantly different from mice treated with endotoxin and 6-MP simultaneously ($P < 0.05$).

placed in ice and rinsed with 0.9% NaCl. These livers were used for assays of enzyme activity and 6-MP-nucleotide. Each group contained three to four mice, and all experiments were carried out in duplicate.

Livers from the mice treated with [8-^{14}C]6-MP were extracted with 1.5 vol. of cold 0.5 M perchloric acid (PCA). These PCA extracts were subjected to the same method as previously described [9], in order to determine TIMP.

Sodium pentobarbital was administered at a dose of 35 mg/kg. Sleeping time was measured by stopwatch as the time from loss of the righting reflex to its restoration. This reflex was defined as the capacity of the mice to simultaneously place both forefeet on to the ground twice within 15 sec.

The livers were homogenized in a Teflon homogenizer with 9 vol. of ice-cold 1 mM Tris buffer of pH 7.5. The homogenates were centrifuged at 30,000 *g* for 20 min at 4°, and the supernatant fractions used for enzyme assay. Enzyme activity was assayed by the same radiochemical methods as previously described [9, 10] for HGPRTase.

The livers were homogenized in a Teflon homogenizer with 9 vol. of cold distilled water, and then used for the assay of xanthine oxidase. Activity was measured by the method of Roussos [11]. The incubation mixture (1.0 ml) consisted of 5 mM glycine buffer at pH 8.8, 0.49 mg albumin, 0.26 mM xanthine, and 0.1 ml of the homogenate. The reaction was stopped after 20 min at 37° by adding 1 ml of 0.5 M PCA. Each sample had its own control tube to which the substrate was added after the addition of 0.5 M PCA. Tubes were centrifuged and the supernatant fractions used for measuring optical density at 290 nm. The increase in optical density was converted to nmoles uric acid formed/min/g of liver. Uric acid formation was shown to have a linear relationship to incubation time and to the amounts of homogenate, under the conditions used.

RESULTS

BALB/c mice which were administered 100 mg 6-MP/kg or 4 mg endotoxin/kg survived the 14-day observation: however, all of the mice which had received 6-MP and endotoxin simultaneously at those doses died within 3 days. The majority of

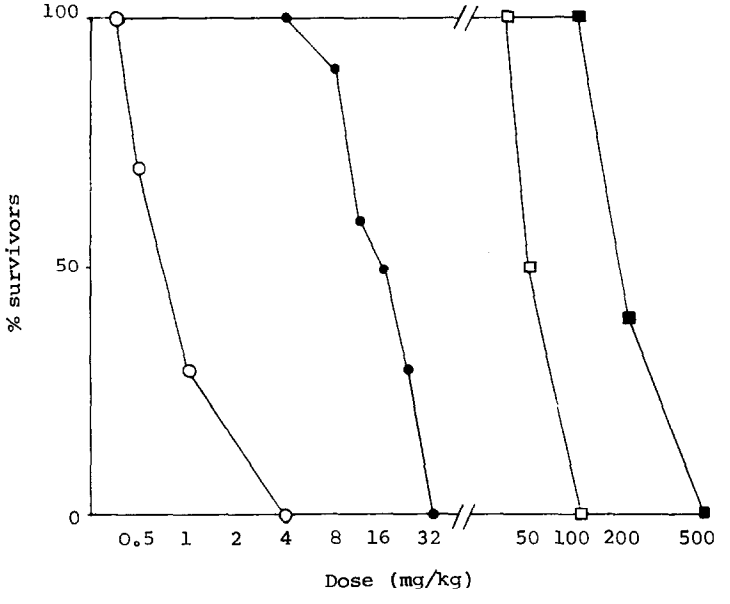

Fig. 1. Three-day survival of mice given various doses of endotoxin with 100 mg 6-MP/kg (○), mice given various doses of 6-MP with 4 mg endotoxin/kg (□), mice given endotoxin alone (●), and mice given 6-MP alone (■). Groups of ten to sixteen mice were used.

deaths resulting from the combination occurred during the first 24 hr after injection. These results show that these agents interact *in vivo*. Deaths due to 500 mg/kg of 6-MP alone occur between 3 and 7 days.

Table 1 shows that, when 4 mg/kg of endotoxin was given at various times before or after 100 mg/kg of 6-MP, the death rate at 3 days was greatest when 6-MP and endotoxin were given simultaneously Endotoxin does not appear to interact either 24 hr before or after 6-MP.

When mice were given 100 mg/kg of 6-MP and various doses of endotoxin simultaneously, increased mortality at 3 days was found at a dose of more than 0.5 mg/kg of endotoxin. Mortality rose progressively as the dose of endotoxin was increased and was complete when 4 mg/kg was given. Endotoxin alone produced no deaths in 3 days unless 8 mg/kg or more was given. Conversely, when various doses of 6-MP were administered simultaneously with 4 mg/kg of endotoxin, no death within 7 days was seen at doses of 25 mg/kg of 6-MP. But mortality rose progressively as the dose of 6-MP was increased, and was complete when 100 mg/kg was given. When 6-MP is given alone, 7-day deaths are not seen at doses below 125 mg/kg. These results suggest that 6-MP and endotoxin significantly potentiate each other in a parallel manner (Fig. 1).

To determine whether treatment with endotoxin would stimulate TIMP formation in mouse liver, mice were given labeled 6-MP with endotoxin. PCA extracts of liver were prepared 0.5, 1, 2 and 3 hr after treatment with 6-MP. As seen in Fig. 2, liver concentrations of TIMP in control mice were maximal 1 hr after 6-MP, and thereafter rapidly declined. When a single dose of 6-MP–endotoxin mixture was injected, the conversion of 6-MP to TIMP was not enhanced 3 hr after treatment. However, it was realized that pretreatment with endotoxin might influence the metabolism of 6-MP in the livers. The concentration of TIMP derived from 6-MP, 6 hr after pretreatment with endotoxin, was significantly

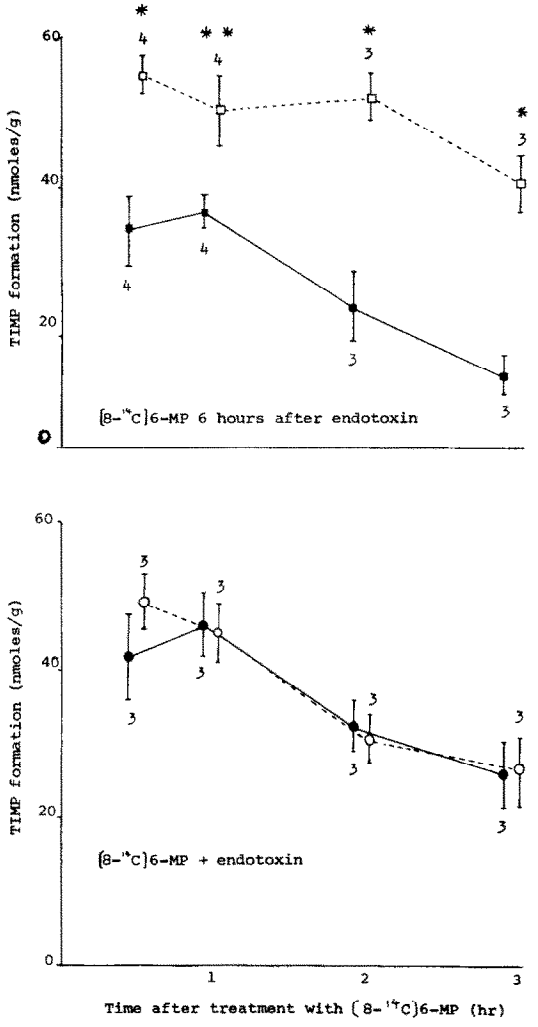

Fig. 2. Time course of TIMP formation in mouse liver after treatment with either [8-^{14}C]6-MP (6 mg/kg) 6 hr after endotoxin (4 mg/kg) (-□-, -■-) or [8-^{14}C]6-MP (6 mg/kg) and endotoxin (4 mg/kg) (-○-, -●-). The mean and S.E. are shown (N = 3 or 4). One asterisk indicates $P < 0.01$; two indicate $P < 0.05$.

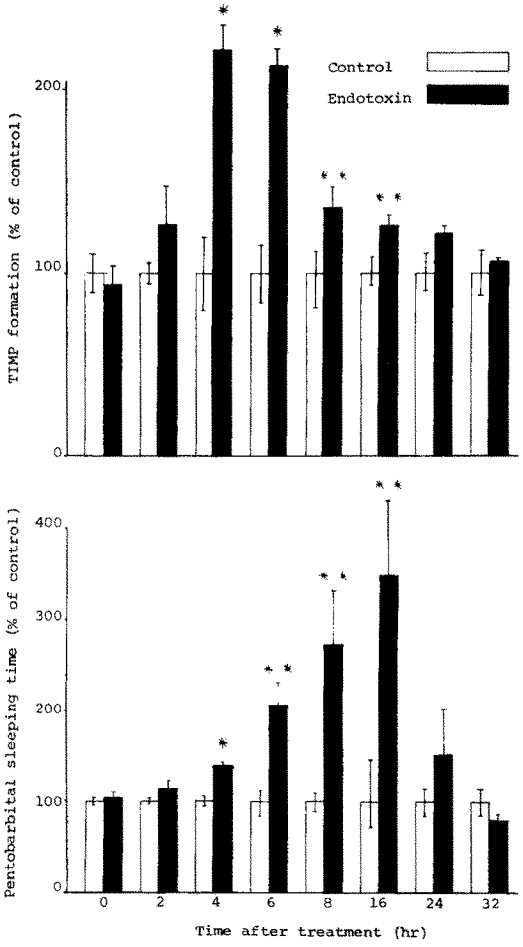

Fig. 3. Effects of endotoxin on TIMP formation and pentobarbital sleeping time. Mice were subjected to the experiments at various times after endotoxin. Mice were killed for TIMP determination 2 hr after injection of [8-^{14}C]6-MP at a dose of 6 mg/kg. The mean and S. E. are shown (N = 4). One asterisk indicates $P < 0.01$; two indicate $P < 0.05$.

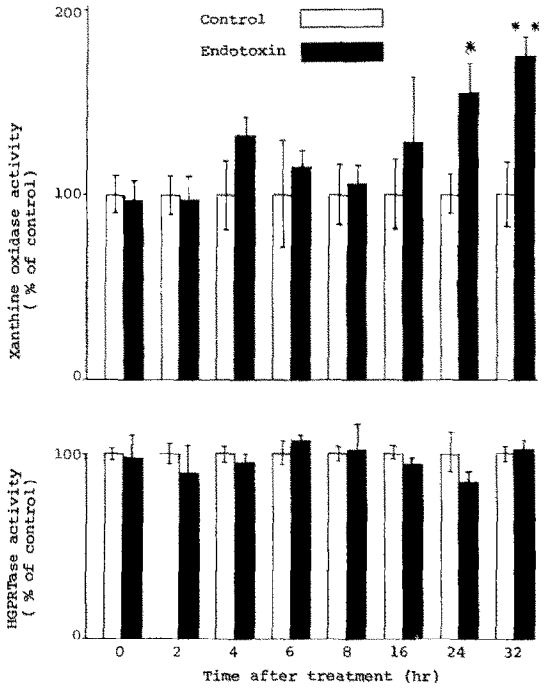

Fig. 4. Effects of endotoxin on HGPRTase and xanthine oxidase activities in mouse livers. Assay conditions are described in Materials and Methods. Endotoxin was administered intraperitoneally at a dose of 8 mg/kg. The mean and S.E. are shown ($N = 4$). One asterisk indicates $P < 0.05$; two indicate $P < 0.01$.

raised to high levels 0.5 to 3 hr after 6-MP injection. To study the time dependence of the endotoxin stimulatory effect, conversion of 6-MP to TIMP was assessed at various times after injection of endotoxin. As shown in Fig. 3, TIMP formation was significantly increased 4–16 hr after endotoxin. We asked whether a change in activity of hepatic microsomal enzymes might lead to a change in the amounts of TIMP formed. It was found that, after pretreatment with endotoxin, sleeping time after pentobarbital was significantly prolonged 4–16 hr after the endotoxin injection. This agrees with the time course of the stimulatory effect of endotoxin on the conversion of 6-MP to TIMP. It is apparent from these results that endotoxin was responsible for the enhancement of the conversion of 6-MP to TIMP. However, this stimulatory effect is not consistent with the lethality determined *in vivo*.

Table 2. Effect on mortality of endotoxin and 6-mercaptopurine of Na HPO and inosine

Treatment	Survivors at 3 days
Na_2HPO_4 (0.15 M/kg)	10/10
Inosine (100 mg/kg)	10/10
Endotoxin (32 mg/kg) after treatment with	
Nothing (control)	0/16
Na_2HPO_4 (0.15 M/kg)	9/16
Inosine (100 mg/kg)	10/16*
6-MP (500 mg/kg) after treatment with	
Nothing (control)	0/16
Na_2HPO_4 (0.15 M/kg)	0/16
Inosine (100 mg/kg)	1/16
Endotoxin (4 mg/kg) + 6-MP (100 mg/kg) after treatment with	
Nothing (control)	0/14
Na_2HPO_4 (0.15 M/kg)	4/13*
Inosine (100 mg/kg)	5/13*

* Significantly different from control ($P < 0.05$).

HGPRTase and xanthine oxidase, which have been found to be involved in 6-MP metabolism, were assayed after endotoxin injection. Approximately the LD_{10}, 8 mg/kg of endotoxin, was injected. Up to 32 hr after the endotoxin injection, there was no significant difference in HGPRTase activity between the injected mice and control mice. Furthermore, contrary to expectation, there were significant differences in xanthine oxidase 24 and 32 hr after treatment compared to controls. These experiments clearly demonstrate that the enhanced toxicity of 6-MP with endotoxin cannot be explained by an alteration in these enzyme activities. Furthermore, enzymes other than xanthine oxidase may be more responsible for the catabolism of 6-MP (Fig. 4).

Administration of phosphate and inosine provides a protective effect to mice challenged with a lethal dose of endotoxin [12, 13]. Inorganic phosphate and inosine was administered 0.5 hr prior to injection of 6-MP-endotoxin combinations. Significant protection was provided against the lethal effects of a combination of 100 mg/kg of 6-MP and 4 mg/kg of endotoxin by inorganic phosphate and inosine. Mice pretreated with inorganic phosphate and inosine were resistant to the high doses of endotoxin, but the toxicity of 6-MP was not altered by inorganic phosphate and inosine (Table 2). These results indicate that death due to 6-MP–endotoxin combinations might rely heavily on an enhancement of the lethal effects of endotoxin.

DISCUSSION

The enhanced toxicity of 6-MP with endotoxin has become well-recognized in mice taking a combination of the agents [4]. When we experimented on mice, our data also confirmed these findings. As to which mechanism is responsible for this interaction, it has not been determined. Three hypotheses have been postulated: (1) endotoxin-mediated alteration in 6-MP metabolism, (2) 6-MP-mediated alteration in endotoxin toxicity, and (3) a dualistic enhancement of each agent. It is very important to determine the events responsible for the enhanced toxicity from the combination of endotoxin and 6-MP, because the patients receiving intense antitumor therapy display an increased susceptibility to bacterial infections [2, 3]. Our studies demonstrate an increase of the conversion of 6-MP to TIMP in the liver by endotoxin administered to mice: this is the first reported evidence of the effect of endotoxin on activation of 6-MP in any experimental animals. Prior treatment of mice with endotoxin enhanced significantly their ability to convert 6-MP to its nucleotide in agreement with the time course of the inhibiting effect of endotoxin on sleeping time. From the data collected in these studies it is clear that metabolism of 6-MP might be modified by activity of the drug-metabolizing enzyme in mouse liver. The

present studies also confirm the result [6] that 6-MP can be catabolized *in vitro* by hepatic microsomal enzyme from rats. However, in the period when increases were observed in the conversion of 6-MP to TIMP after endotoxin injection, 6-MP injection did not result in a significant increase in deaths with respect to simultaneous administration of the same respective dosages. When a single dose of 6-MP–endotoxin mixture was injected, the conversion of 6-MP to TIMP was not enhanced in spite of its enhanced toxicity. Although endotoxin might enhance the conversion of 6-MP to TIMP in mouse liver, these effects are not large enough to explain the mechanism of interaction. These results indicate that the tumor-inhibitory effect is due to TIMP, but that overall toxicity might not be due to this compound.

Mice dying from endotoxin alone or in combination with 6-MP die within 24 hr after administration. However, death due to 6-MP alone occurs between 3 and 7 days. Based on time of death, it is proposed that death caused by 6-MP–endotoxin combinations is due to an enhancement of the lethal effects of endotoxin.

Based on dose–response curves, the underlying mechanism of the interaction may involve a dualistic enhancement of each agent. If this is a correct interpretation, it is reasonable to assume that the interaction would be abolished in mice resistant to the effect of endotoxin. Mice pretreated with inorganic phosphate and inosine do not respond to the 6-MP–endotoxin combination. Conversely, endotoxin is no longer able to potentiate the lethal action of 6-MP. The experiments reported here show that the interaction between 6-MP and endotoxin, therefore, appears to be due to a mutual enhancement of the lethal action of each agent. Our data are not inconsistent with the last proposal.

Since high doses of 6-MP were used to investigate lethality, the physiologic or pharmacologic significance is not apparent clinically [14]. It is important to note whether the levels of endotoxin administered are comparable to those levels commonly present in patients with bacteremia [15]. An effort needs to be made to resolve these problems.

REFERENCES

1. G. B. Elion, *Fedn Proc.* **26,** 898 (1967).
2. E. M. Hersh, G. P. Bodey, B. A. Nies and E. J. Freireich, *J. Am. med. Ass.* **193,** 105 (1965).
3. E. A. Sickles, V. H. Young, W. H. Green and P. H. Wiernik, *Ann. intern. Med.* **79,** 528 (1973).
4. N. M. Marecki and S. G. Bradley, *Antimicrob. Agents Chemother.* **5,** 413 (1974).
5. R. Gorodischer, J. Krasner, J. J. McDeritt, J. P. Nolan and S. J. Yaffe, *Biochem. Pharmac.* **25,** 351 (1976).
6. J. Yoshimura, in *Proc. 34th A. M. Jap. Cancer Ass. Tokyo*, p. 145 (1975).
7. F. S. Philips, S. S. Sternberg, L. Hamilton and D. A. Clarke, *Ann. N.Y. Acad. Sci.* **60,** 184 (1954).
8. D. F. Rippe, J. G. Saltas and L. J. Berry, *J. reticuloendothel. Soc.* **16,** 175 (1974).
9. T. Higuchi, T. Nakamura and G. Wakisaka, *Cancer Res.* **36,** 3779 (1976).
10. T. Higuchi, T. Nakamura and G. Wakisaka, *Biochem. Med.* **13,** 178 (1975).
11. G. G. Roussos, in *Methods in Enzymology* Vol. XII, p. 5. Academic Press, New York (1968).
12. A. R. Kraft, W. Peterson, L. D. Homer, R. B. Jones, L. Keisow and C. M. Herman, *Surg. Forum* **25,** 19 (1974).
13. C. H. Baker, *Proc. Soc. exp. Biol. Med.* **141,** 694 (1972).
14. E. J. Freireich, E. A. Gehan, D. P. Rall, L. H. Schmidt and H. E. Skipper, *Blood* **21,** 699 (1963).
15. S. M. Wolff, *J. infect. Dis.* **128,** s. 259 (1974).