APPLIED MICROBIOLOGY, July, 1965
Vol. 13, No. 4

In Vivo Antiviral Activity of 1,3-Bis(2-Chloroethyl)-1-Nitrosourea

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Received for publication 5 March 1965

ABSTRACT

SIDWELL, ROBERT W. (Southern Research Institute, Birmingham, Ala.), GLEN J. DIXON, SARA M. SELLERS, AND FRANK M. SCHABEL, JR. In vivo antiviral activity of 1,3-bis(2-chloroethyl)-1-nitrosourea. Appl. Microbiol. **13**:579–589. 1965.—A prolongation in the lives of Swiss mice inoculated intracerebrally with lymphocytic choriomeningitis virus (LCM) was observed after treatment with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). A variety of treatment schedules, including therapy once or twice daily up to 17 days and single treatments at various times after virus inoculation, were employed. Virus titers ranging to greater than 10^4 were detected in the blood and brains of surviving drug-treated animals. In three comparative studies in which different treatment schedules were used, BCNU was shown to exert a protective effect approximately equal to that of methotrexate in LCM virus-infected mice. Tests were also carried out to investigate the activity of BCNU in mice experimentally infected with eastern equine encephalomyelitis (EEE) virus, western equine encephalomyelitis virus, Semliki Forest (SF) virus, herpes simplex virus, influenza virus strain PR8, vaccinia virus strain WR, Rous sarcoma virus, Friend leukemia virus (FLV), and poliovirus. Slight increases in life span were observed in the treated EEE, SF, and influenza PR8 virus-infected animals. Significant reduction in splenomegaly in FLV-infected animals treated with BCNU was demonstrated. The possible mechanisms of LCM virus inhibition by BCNU, on the basis of these and other studies, were postulated to be either specific antiviral activity or inhibition of "lethal" immune response to the LCM virus. Each of these postulates is discussed.

The class of compounds known as the alkylating agents had not been used extensively for in vivo antiviral drug testing until recently, when definite activity was demonstrated with a number of these chemicals in systems using certain animal leukemia viruses. For example, Cytoxan, triethylenemelamine (TEM), and Melphalan were used effectively against the Rauscher murine leukemogenic virus (Chirigos et al., 1963). The chemical name for Cytoxan (cyclophosphamide) is 2H-1,3,2-oxazaphosphorinane, 2-[bis(2-chloroethyl)amino]-2-oxide; that of Mephalan is ^2p-di-(2-chloroethyl)amino-L-phenylalanine. Cytoxan and TEM were also reportedly active against the Moloney leukemia virus (Chirigos et al., 1961). Antiviral activity was demonstrated against the Friend leukemia virus by mitomycin C (Sugiura, 1960) and porfiromycin (Sidwell, Dixon, and Sellers, 1965).

A recently synthesized nitrosourea, 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU), has been shown to have a primary mechanism of action related to that of the alkylating agents (Schabel et al., 1963). Previously, BCNU was effectively used against intracerebrally or intraperitoneally (ip) inoculated leukemia L1210 (Schabel et al., 1963) and is, therefore, presumably capable of passing the blood-brain barrier. Radioisotope-labeled fragments of the drug have been recovered in the brain, as well as in other organs of the host (Wheeler, Bowdon, and Herren, 1964). Because of this probable capability of crossing the blood-brain barrier, in addition to the demonstrated activity against L1210 mouse leukemia and Rauscher murine leukemogenic virus (Chirigos, 1964), it was of interest to determine the activity of this compound against a broad spectrum of viruses inoculated by a variety of routes. These viruses include: lymphocytic choriomeningitis (LCM), eastern equine encephalomyelities (EEE), western equine encephalomyelitis (WEE), Semliki Forest (SF), herpes simplex (HS), polio, vaccinia strain WR, influenza A strain PR8, Friend leukemia (FLV), and Rous sarcoma (RSV). This report describes extensive studies carried out against the LCM virus and limited experiments with the other viruses cited.

MATERIALS AND METHODS

Laboratory animals. Young adult, random-bred Swiss mice weighing 18 to 21 g were employed as

test animals in all studies except those with influenza virus and RSV. In tests with influenza fluenza virus, 8- to 10-g Swiss mice were used. Male Heisdorf-Nelson white leghorn chicks, 10 days old, were used to test the activity of the drug against RSV.

Viruses. Viruses used in these studies were obtained as follows: LCM virus strain M7, EEE virus strain ME8, WEE virus strain M28, and poliovirus type 2 strain MEF-1 from W. A. Rightsel, Parke, Davis & Co., Detroit, Mich.; SF virus "original strain" (ATCC VR67) from the American Type Culture Collection; HS virus strain 123, originally isolated from a human herpetic keratitis lesion, from R. E. Francis of the University of Alabama Medical Center, Birmingham; vaccinia virus strain WR (ATCC VR119) from Arthur Brown, Fort Detrick, Frederick, Md.; influenza A virus strain PR8 from Bernice Eddy, Laboratory of Virology and Rickettsiology, National Institutes of Health, Bethesda, Md.; FLV from H. Christine Reilly, Sloan-Kettering Institute for Cancer Research, Rye, N.Y.; and RSV from W. R. Bryan, National Cancer Institute, Bethesda, Md.

The LCM, EEE, WEE, SF, and vaccinia WR viruses were received in mouse-brain suspension. Each was passed once intracerebrally through mice prior to use. When received, the poliovirus was in monkey-kidney cell suspension and was used in this form. The HS virus, received in human amnion cell suspension, was passed five times through primary human amnion cell cultures. The influenza PR8 virus was obtained in lyophilized mouse lung. This material was reconstituted and passed intranasally five times through 8- to 10-g mice prior to use. The FLV was received in 10% mouse spleen suspension and given a single ip passage through mice. The spleens from these mice were homogenized in Locke-Ringer's solution and filtered through Selas 03 filters. This spleen filtrate material was employed for antiviral drug testing. The RSV was provided as stable,

TABLE 1. *Effect of 1,3-bis(2-chloroethyl)-1-nitrosourea, administered according to treatment schedule 1,* upon lymphocytic choriomeningitis virus infections in mice*

Drug dose (mg per kg per day)	Virus dose (LD_{50})	Drug control mortality (%)	Test mice (survivors/total)	Survivor P †	Mean survival of mice dying on or before final day (days)	Mean survival P ‡
11.3	10	50	2/10	<0.3	17.9	<0.001
7.5		40	2/10	<0.3	13.9	<0.001
5.0		0	0/10	—	9.1	—
3.4		0	0/10	—	11.2	<0.05
2.2		10	0/10	—	10.0	>0.05
0			0/10		9.1	
11.3	32	50	1/10	>0.5	15.9	<0.001
7.5		40	1/10	>0.5	11.2	<0.001
5.0		0	0/10	—	10.6	<0.001
3.4		0	1/10	>0.5	9.1	<0.001
2.2		10	0/10	—	8.2	—
0			0/10	—	8.3	
11.3	10	40	0/10	—	15.9	<0.001
7.5		0	3/10	—	15.7	<0.001
5.0		0	2/10	—	11.5	>0.05
3.4		0	2/10	—	9.1	—
2.2		0	3/10	—	9.6	>0.05
0			4/10		9.3	
11.3	32	40	0/10	—	18.3	<0.001
7.5		0	0/10	—	16.3	<0.001
5.0		0	1/10	—	14.8	<0.001
3.4		0	1/10	—	9.9	>0.05
2.2		0	0/10	—	10.1	>0.05
0			1/10		9.7	

* Treatment schedule 1: drug injected ip once daily for 15 days beginning 24 hr after virus inoculation. Duration of all experiments was 21 days after virus inoculation.

† Probability that observed increase in number of survivors in the treated groups compared with the virus control group was due to chance. Determined by chi-square analysis.

‡ Probability that observed increase in the mean survival of the drug-treated groups compared with the virus control group was due to chance. Determined by *t* test.

standardized virus prepared from chicken tumor tissue by differential centrifugation (Bryan, Moloney, and Calnan, 1954) and was used in the form received. For use in drug evaluations, stock RSV was diluted in Eagle's (1955) balanced salt solution containing 1% heat-inactivated horse serum. All the above viruses were stored at the temperature of solid CO_2 in sealed glass ampoules.

Compound. The BCNU used for these studies was synthesized at Southern Research Institute. This compound is unstable when in phosphate-buffered saline and thus was always dissolved in sterile neutral physiological saline and administered to test animals within 15 min after preparation. The 21-day LD_{10} of this compound in Swiss mice is approximately 5.0 mg per kg per day when injected ip once daily for 15 days, and 24.0 mg/kg when administered ip in a single injection.

Plan of experiment. At each drug dosage in all experiments, 10 animals were injected with virus, and 10 animals received virus diluent and were used as drug toxicity controls. A total of 10 to 20 animals, injected with virus but treated with saline only, were employed as virus controls. All mice were held 2 to 6 weeks, according to the test carried out, after which the survivors were killed.

Two methods were used for evaluating activity against all viruses except FLV and RSV. The number of test mice which survived virus infection after drug treatment was compared with the number of survivors in the untreated control group. The chi-square analysis technique was used to determine the statistical significance of any observed increase in the number of survivors. A mean survival time was then determined in each treatment group of mice dying on or before the final day of the experiment. The significance of an increased mean survival time of drug-treated, virus-infected animals, as compared with the mean survival time of the virus controls, was determined by the *t* test.

To demonstrate the activity of the drug against FLV, the animals were killed 14 days after virus inoculation and their spleens were removed and weighed, since FLV induces a marked splenomegaly by this time. A reduction in splenomeg-

TABLE 2. *Effect of 1,3-bis(2-chloroethyl)-1-nitrosourea, administered according to treatment schedule 2,* upon lymphocytic choriomeningitis virus infections in mice*

Drug dose (mg per kg per day)	Expt duration (days after virus inoculation)	Virus dose (LD_{50})	Drug control mortality (%)	Test mice (survivors/total)	Survivor *P*†	Mean survival of mice dying on or before final day (days)	Mean survival *P*‡
11.3	21	10	50	1/10	—	14.9	<0.001
7.5			30	3/10	>0.5	10.3	>0.05
5.0			10	5/10	<0.2	15.4	<0.001
3.4			0	5/10	<0.2	12.4	<0.001
2.2			0	4/10	<0.3	9.7	>0.05
0				1/10		9.2	
11.3	21	32	50	1/10	>0.5	17.7	<0.001
7.5			30	1/10	>0.5	19.2	<0.001
5.0			10	5/10	<0.05	10.6	<0.05
3.4			0	2/10	<0.3	10.1	<0.05
2.2			0	1/10	>0.5	9.4	>0.05
0				0/10		8.4	
15.0	41	10	90	0/10	—	14.0	<0.001
10.0			50	0/10	—	21.7	<0.001
7.7			40	1/10	>0.5	17.2	<0.001
4.4			20	0/10	—	10.4	<0.001
0				0/20		8.4	
15.0	41	32	90	0/10	—	17.8	<0.001
10.0			50	0/10	—	12.3	>0.05
7.7			40	0/10	—	16.4	<0.001
4.4			20	0/10	—	10.5	>0.05
0				0/20		9.9	

* Treatment schedule 2: drug injected ip once daily for 15 days beginning 24 hr prior to virus inoculation.

† Probability that observed increase in number of survivors in the treated groups compared with the virus control group was due to chance. Determined by chi-square analysis.

‡ Probability that observed increase in mean survival in treated groups compared to the virus control group was due to chance. Determined by *t* test.

aly due to treatment with drug was determined by calculating, for each drug dosage, the percentage of virus control average spleen weight. The reduction in spleen weight was then evaluated statistically by use of the *t* test. To determine anti-RSV activity, test chickens were injected with virus subcutaneously in the wing web, treated with drug, and killed on day 10. The degree of inhibition in tumor weight as compared with virus control chicks was then calculated.

Therapy schedules for treatment of LCM virus infections. BCNU was tested against LCM virus-infected mice with the following therapy schedules. Treatment schedule 1: drug injections were given ip once daily, beginning 24 hr after virus inoculation and continuing for 15 days. Treatment schedule 2: drug injections were given ip once daily, beginning 24 hr before virus inoculation and continuing for 17 days. Treatment schedule 3: a single drug injection was given ip 24 hr after virus inoculation. Treatment schedule 4: a single drug injection was given ip 4 hr after virus inoculation. Treatment schedule 5: a single drug dosage was given orally 4 hr after virus inoculation.

In addition to the above, studies were carried out in which a single drug injection was given ip 24 or 100 hr after virus inoculation, with BCNU and methotrexate (MTX) being compared in the same experiment. The chemical name for MTX (amethopterin) is *N*-{*p*[(2,4 - diamino-6-pteridinyl-methyl) methylamino] benzoyl} glutamic acid. These drugs were also compared by use of treatment schedule 1.

RESULTS

Effect of BCNU upon LCM virus infections. Treatment schedule 1 (Table 1) was used in two experiments with drug dosages of 2.25, 1.5, 1, 0.67, and 0.44 LD_{10}. Two virus concentrations, 10 and 32 LD_{50}, were employed for each drug dose. Test animals infected with 10 LD_{50} of virus and treated with the two highest doses were still alive by 21 days, with an observed average life span of 6 to 9 days more than the virus control animals. Fewer survivors, but significantly increased life spans, were demonstrated among BCNU-treated mice infected with 32 LD_{50} of LCM virus. Virtually identical results were observed with this treatment schedule in a second experiment.

Treatment schedule 2 (Table 2) was tested with the same doses of virus and the same daily dosages of drug as described above. With this schedule, in which treatment began 24 hr before

TABLE 3. *Effect of 1,3-bis(2-chloroethyl)-1-nitrosourea, administered according to treatment schedule 3,* upon lymphocytic choriomeningitis virus infections in mice*

Drug dose (mg per kg per day)	Virus dose (LD_{50})	Drug control mortality (%)	Test mice (survivors/total)	Survivor P†	Mean survival of mice dying on or before final day (days)	Mean survival *P*‡
90.0	10	90	1/10	>0.5	5.9	—
60.0		60	4/10	<0.3	12.3	<0.001
40.0		20	6/10	<0.1	11.0	<0.001
26.8		0	2/10	>0.5	10.1	<0.001
17.6		10	0/10	—	10.2	<0.001
0			1/10		8.9	
90.0	10	100	0/10	—	5.7	—
60.0		90	0/10	—	8.2	—
40.0		40	6/10	<0.2	12.7	<0.001
26.8		0	5/10	>0.3	10.8	<0.001
17.6		0	6/10	<0.2	12.5	<0.001
0			2/10		8.9	
90.0	32	100	0/10	—	5.9	—
60.0		90	3/10	<0.3	8.0	—
40.0		40	3/10	<0.3	15.9	<0.001
26.8		0	2/10	>0.3	14.6	<0.001
17.6		0	1/10	>0.5	10.6	<0.001
0			0/10		8.2	

* Treatment schedule 3: drug injected ip once only, 24 hr after virus inoculation. Duration of all experiments was 21 days after virus inoculation.

† Probability that observed increase in number of survivors in the treated groups compared with the virus control group was due to chance. Determined by chi-square analysis.

‡ Probability that observed increase in mean survival in treated groups compared with the virus control group was due to chance. Determined by *t* test.

TABLE 4. *Effect of 1,3-bis(2-chloroethyl)-1-nitrosourea, administered according to treatment schedule 4[a] upon lymphocytic choriomeningitis virus infections in mice*

Drug dose (mg/kg)	Expt duration (days after virus inoculation)	Virus dose (LD_{50})	Drug control mortality (%)	Test mice (survivors/total)	Survivor P^b	Mean survival of mice dying on or before final day (days)	Mean survival P^c	Virus titer in blood and brain of survivors[d]
33.0	35	100	20	0/10	—	12.3	<0.001	
30.0			10	0/10	—	13.0	<0.001	
27.0			40	0/10	—	10.9	<0.001	
24.0			30	0/10	—	15.7	<0.001	
0				0/20		7.6		
30.0	17	10	0	1/20	>0.5	10.7	<0.01	Bl = $>10^4$; Br = 10^3
27.0			0	2/20	<0.3	11.2	<0.001	Bl = 10^0, 10^2; Br = $<10^1$, $>10^5$
0				0/30		8.1		
30.0	17	32	0	2/20	<0.3	10.4	<0.05	Bl = $>10^4$, 10^2; Br = $>10^5$, $>10^5$
27.0			0	1/20	>0.5	10.1	<0.05	Bl = $>10^4$; Br = $>10^5$
0				0/30		8.3		

[a] Treatment schedule 4: drug injected ip once only, 4 hr after virus inoculation.

[b] Probability that observed increase in number of survivors in the treated groups compared with the virus control group was due to chance. Determined by chi-square analysis.

[c] Probability that observed increase in mean survival in treated groups compared with the virus control group was due to chance. Determined by *t* test.

[d] All surviving virus-infected mice were sacrificed on day 17, and the virus titer in the blood (Bl) and brain (Br) was determined by intracerebral inoculation into 18 to 21-g indicator Swiss mice.

virus inoculation, significant increases in life span again were demonstrated among drug-treated groups. At the end of 21 days as many as 50% of the treated animals were still alive, the increases in life span varying from 2 to 11 days beyond that of the virus controls. Significant prolongation of live span was noted. The experiment was repeated with the identical treatment schedule but with slight alterations in drug doses. This second experiment ended 41 days after virus inoculation, with significant increases in life span again demonstrated, although only one virus-infected animal survived the duration of the experiment. Drug-treated animals died late in the study, indicating a delayed drug toxicity, and subsequently causing the drug control mortality to be higher than in the first experiment with this treatment schedule.

Two separate experiments were carried out with treatment schedule 3 (Table 3). In the first study, mice infected with 10 LD_{50} of the virus were treated ip with 90.0, 60.0, 40.0, 26.8, and 17.6 mg/kg of BCNU. The first three doses were in excess of the calculated LD_{10}, but as many as 60% of the treated, virus-infected mice were still alive at the end of the experiment. A repetition of the experiment, using 10 and 32 LD_{50} of virus and the same drug dosages, yielded similar results at nontoxic drug levels.

In an experiment with treatment schedule 4 (Table 4), mice were infected with 100 LD_{50} of LCM virus and treated in a single injection 4 hr after virus inoculation with dosages of BCNU arranged near a toxic level. The animals were held 35 days; none of those infected with virus survived for the duration of the experiment, although significant increases in life span occurred. This experiment was repeated with 10 and 32 LD_{50} of virus and two drug dosages. Six of the treated mice survived the infection. These animals were sacrificed on day 17, and the virus level in the blood and brain was determined by injecting dilutions of the bacteriologically sterile material intracerebrally into 18- to 21-g Swiss mice. Death in these animals, 3 to 16 days later, was considered indicative of presence of virus. Control animals injected at the same time with blood and brain from uninfected mice survived through 24 days postinoculation. Blood from the surviving virus-infected mice treated with BCNU contained virus in titers ranging to greater than 10^4. Similar virus titers were also observed in the brains from these animals.

With treatment schedule 5 (table 5), four dosages of BCNU were used orally against 10

TABLE 5. *Effect of oral 1,3-bis(2-chloroethyl)-1-nitrosourea, administered according to treatment schedule 5,* upon lymphocyti choriomeningitis virus infections in mice*

Drug dose (mg/kg)	Virus dose (LD_{50})	Drug control mortality (%)	Test mice (survivors/total)†	Mean survival of mice dying on or before final day (days)	Mean survival P‡
22.0	10	0	0/10	10.7	<0.05
19.0		10	1/10	8.0	—
16.0		10	1/10	8.4	—
13.0		0	0/10	10.4	>0.05
0			4/20	8.6	
22.0	32	0	0/10	10.2	<0.001
19.0		10	0/10	8.2	>0.05
16.0		10	0/10	8.5	>0.05
13.0		0	0/10	8.8	>0.05
0			1/20	8.0	

* Treatment schedule 5: drug administered orally once only, 24 hr after virus inoculation. Duration of both experiments was 32 days.

† Probability that observed increase in number of survivors in the treated groups compared with the virus control group was due to chance, determined by chi-square analysis, was not significant.

‡ Probability that observed increase in mean survival in treated groups compared with the virus control group was due to chance. Determined by t test.

and 32 LD_{50} of virus. The animals were held 32 days after virus inoculation. Significant increases in mean survival time could be demonstrated at the highest drug dosage only.

Comparison of the antiviral activities of MTX and BCNU. It has been reported that MTX, a folic acid analogue, unequivocally increases the life span of mice infected with LCM virus (Haas and Stewart, 1956). Treatment of LCM virus-infected mice with other compounds, including 8-azaguanine, azaserine, 6-diazo-5-oxo-L-norleucine, 5-fluorouracil, chlorambucil, and cortisone, also increases the average life span, although to a lesser extent than MTX (Haas and Stewart, 1956; Levy and Haas, 1958; Barlow, 1962; Hotchin, 1962). To compare the anti-LCM virus activities of BCNU and MTX, three virus concentrations (32, 100, and 1,000 LD_{50}) were used. The drugs were administered in a single injection ip 24 or 100 hr after virus inoculation. Four dosages of BCNU and MTX were used against the 32 LD_{50} virus level; a single dose of MTX was used against the 100 LD_{50} virus level, and three dosages were tested against the 1,000 LD_{50} inoculum of virus. The dosages of MTX used for the 100 hr postinoculation treatment were in the range of those described by Hotchin (1962) as being very effective when administered under the conditions used in this study. The results (Table 6) indicated that both compounds were effective against the LCM virus when administered 100 hr after virus inoculation. No significant antiviral activity could be demonstrated with MTX administered 24 hr after virus inoculation, whereas 40% of the BCNU-treated mice survived at the highest drug level given at this same time, and the mean survival time was lengthened at the two highest levels of BCNU. An experiment was also done to determine the comparative anti-LCM virus activities of the two compounds administered once daily for 15 days, beginning 24 hr after virus inoculation (Table 7). Both BCNU and MTX significantly increased the mean survival time of the virus-infected mice; 10 to 50% of the mice survived the duration of the experiment when treated with slightly toxic dosages of MTX, although the same percentages of survivors occurred in the previously discussed experiments with BCNU and with similar schedules (Tables 1 and 2).

Effect of BCNU upon infections with other viruses. A number of experiments were carried out to determine the in vivo antiviral activity of BCNU upon the other previously described viruses. The results are summarized in Tables 8 and 9. Various treatment schedules were employed for these studies, as indicated in the tables. The doses were selected in most tests to vary from toxic to nontoxic levels. Possible antiviral activity was noted against four viruses—EEE, SF, influenza PR8, and FLV—in animals treated chronically with BCNU. In two tests with EEE virus-infected mice, a significant number survived for the duration of the experiment. In two additional experiments carried out to confirm these observations, the observed maximal increase was small, however, and the activity of BCNU against this virus was considered marginal. Increases in mean survival time of SF virus-infected mice treated with BCNU were observed, but this activity also was not seen in later tests; hence, activity against this virus may be questioned. Increases in life span of up to 2 days were observed in mice infected with the lower dose (100 LD_{50}) of PR8 virus and treated with all nontoxic dosages of BCNU, but no activity could be demonstrated in treated mice receiving 320 LD_{50} of virus.

When used against FLV-infected mice, BCNU treatment resulted in a significant decrease in splenomegaly (Table 9), but the average spleen weight of the treated animals was slightly

TABLE 6. *Comparison of the effect of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and methotrexate (MTX), administered in a single treatment, upon lymphocytic choriomeningitis virus infections in mice*

Drug	Drug dose (mg/kg)	Time of treatment* (hr after virus inoculation)	Expt duration (days after virus inoculation)	Virus dose (LD_{50})	Drug control mortality %	Test mice (survivors/total)	Survivor P†	Mean survival of mice dying on or before final day (days)	Mean survival P‡
BCNU	35.0	100	34	100	30	0/10	—	14.4	<0.001
	33.0				30	0/10	—	12.9	<0.001
	30.0				20	1/10	—	12.6	<0.001
	27.0				20	1/10	—	9.9	<0.05
MTX	6.0				0	0/9	—	11.1	<0.001
Saline	0					1/20		8.2	
BCNU	35.0	100	34	1,000	10	0/10	—	15.3	<0.001
	33.0				10	0/10	—	10.0	<0.001
	30.0				0	0/10	—	9.7	<0.001
	27.0				0	0/10	—	9.2	<0.05
MTX	16.0				0	0/10	—	8.9	>0.05
	8.0				0	0/10	—	9.3	<0.05
	4.0				0	1/10	>0.3	9.9	<0.001
Saline	0				0	0/20		7.8	
BCNU	48.0	24	21	32	10	4/10	<0.05	10.8	<0.001
	24.0				20	1/10	>0.3	10.3	<0.001
	12.0				0	0/10	—	8.5	>0.05
	6.0				0	0/10	—	8.5	>0.05
MTX	48.0				90	0/10	—	5.4	—
	24.0				50	0/10	—	6.2	—
	12.0				20	0/10	—	8.4	>0.05
	6.0				0	0/10	—	7.3	—
Saline	0					0/20		8.1	

* Drug injected ip once only 24 or 100 hr after virus inoculation.

† Probability that the observed increase in the number of survivors in the treated groups compared with the virus control group was due to chance. Determined by chi-square analysis.

‡ Probability that the observed increase in mean survival in treated groups compared to the virus control group was due to chance. Determined by t test.

greater (ca. 25%) than the spleen weight of normal Swiss mice.

DISCUSSION

As discussed previously, a number of agents have reportedly shown definite anti-LCM virus activity in certain in vivo systems, although, as pointed out by Levy and Haas (1958), MTX consistently gave better protection against this virus than the other drugs tested. It is noteworthy that, in the experiments reported here, BCNU has exhibited comparable activity to MTX in similar in vivo test systems. Seldom, however, did as many treated, virus-infected animals survive as had occurred in the MTX studies reported by others (Haas and Stewart, 1956; Levy and Haas, 1958; Lerner and Haas, 1958; Hotchin, 1962). This variation in results may have been due to a number of factors, among which may be the strain of LCM virus, the strain of mice, the dose of virus employed, timing of the drug administration, or variations in the drug lot of MTX.

Little is known of the mechanism of action of BCNU, although from available evidence two mechanisms may be speculated: first, that the activity demonstrated is a specific antiviral action and, second, that the prolongation of life in animals treated with BCNU is a result of inhibition of a "lethal" immune response to the LCM virus.

Limited evidence is available in support of the first premise, i.e., that BCNU is a true antiviral compound. Two characteristics of this compound are high lipid solubility and a low ionization constant (Schabel et al., 1963), and it has been postulated that there is a relationship between these properties and the capacity of parenterally administered compounds to cross the blood-brain barrier and subsequently affect an intracerebrally inoculated agent (Rall and Zubrod, 1962). The mechanisms of action of

TABLE 7. *Comparison of the effect of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and methotrexate (MTX), administered chronically,[a] upon lymphocytic choriomeningitis virus infections in mice*

Drug	Drug dose (mg/kg)	Expt duration (days after virus inoculation)	Virus dose (LD_{50})	Drug control mortality (%)	Test mice (survivors/total)	Survivor P^b	Mean survival of mice dying on or before final day (days)	Mean survival P^c
BCNU	7.5	21	100	40	0/10	—	12.9	<0.001
	3.8			10	0/10	—	11.5	<0.001
	1.9			0	0/10	—	8.4	>0.05
	0.9			0	0/10	—	7.6	>0.05
Saline	0				0/20		7.6	
MTX	2.0	21	100	100	0/10	—	10.9	<0.05
	1.0			40	5/10	<0.05	14.0	<0.001
	0.5			10	1/10	>0.3	14.2	<0.001
	0.3			0	0/10	—	9.1	>0.05
CMC[d]	0				1/20		8.5	

[a] Drugs injected ip once daily for 15 days beginning 24 hr after virus inoculation.

[b] Probability that the observed increase in the number of survivors in the treated groups compared with the virus control group was due to chance. Determined by chi-square analysis.

[c] Probability that the observed increase in the mean survival in treated groups compared with the virus control group was due to chance. Determined by *t* test.

[d] Carboxymethylcellulose (0.4%) in phosphate-buffered saline.

alkylating agents, as reviewed by Wheeler (1962) and by Warwick (1963), suggest that these agents would be capable of exerting possible antiviral action. The fact that certain other alkylating agents studied have shown antiviral activity in varying degrees would add weight to this premise. However, since surviving drug-treated mice in the present studies showed evidence of continued viral multiplication, the sparing effect rendered by treatment with BCNU may not be due to reduction or eradication of the virus. No evidence is yet available, however, to indicate whether the surviving virus observed in these studies was resistant to BCNU.

A similar occurrence, in which the host was spared but the virus continued to propagate, has been reported to have taken place in a cell culture system (Furusawa, Cutting, and Furst, 1963). It was observed that several compounds appeared to be effective, since cytopathogenic effect was prevented in Ehrlich ascites cells infected with LCM virus, but virus in high titer was continuously recovered.

The hypothesis that the observed activity of BCNU is a result of suppression of a "lethal" immune response is supported by a number of facts, all primarily based upon the observed anti-LCM virus activity exerted by other compounds. Levy and Haas (1958) speculated that the anti-LCM virus activity of MTX was due to some biochemical effect, although a possible secondary effect upon the host was also suggested. Hotchin (1962) further advanced the latter theory, indicating that a tolerance-inducing mechanism was the probable cause of the observed increased life spans. The host's immune mechanisms were thought to be depressed at a critical time during the immune response, or at least that the "learning ability" of the immune systems during the critical stages of its response to the LCM antigen was impaired. In this way an active immunity was inhibited and a tolerance to the virus resulted. The latter concepts were first stimulated and later strengthened when whole-body X irradiation (Rowe, 1956), folic acid deficiency (Haas, Briggs, and Stewart, 1957*a*, Haas, Stewart, and Briggs, 1957*b*), chlorambucil (Barlow, cited by Hotchin, 1962), and cortisone (Hotchin, 1962), all immune response depressants, produced a mutual host-LCM virus survival. Certain of the alkylating agents (chlorambucil and myleran) apparently also can reproduce the effects of whole body X irradiation on the number of circulating platelets, lymphocytes, and neutrophils, and upon circulating antibodies (Elson, 1958; Elson, Galton, and Till, 1958; Berenbaum, 1962).

Evidence against the latter premise of immune response suppression may be indicated in recent studies by Humphreys, Glynn, and Goldin (1963 and *personal communication*), in which neither BCNU nor MTX appeared to be as effective as X irradiation or alkylating agents such as Cytoxan, TEM, and Melphalan in the suppres-

TABLE 8. *Effect of 1,3-bis(2-chloroethyl)-1-nitrosourea upon eastern equine encephalomyelitis (EEE), western equine encephalomyelitis (WEE), semliki Forest (SF), herpes simplex (HS), polio type 2, influenza (PR8), and vaccinia (WR) virus infections in mice*

Virus	Virus[a] dose (LD_{50})	Drug dose range (mg per kg per day)	Treatment schedule (relative to virus inoculation)	Expt duration (days)	Test mice[b] Maximal per cent survivors (T/C)	Maximal survivor P^c	Maximal increase in mean survival of mice dying on or before final day (days)	Maximal mean survival P^d
EEE	10	5.3–27.0	Once daily 1 through 7 days	15	10/30	>0.3	0.7	<0.05
	32	5.3–27.0		15	0/0	—	0.7	<0.05
	10	5.0–40.0	Once only 24 hr post	26	40/20	>0.3	0.6	>0.05
	32	5.0–40.0		26	30/0	<0.1	0.2	>0.05
	10	5.0–40.0	Once only 4 hr post	26	40/20	>0.3	0.8	>0.05
	32	5.0–40.0		26	30/0	<0.1	0.1	>0.05
WEE	10	21.0–30.0	Once only 24 hr post	31	0/10	—	0.1	>0.05
	32	21.0–30.0		31	10/0	>0.3	0.4	>0.05
SF	10	5.3–27.0	Once daily 1 through 7 days	15	20/10	>0.5	1.8	<0.05
	32	5.3–27.0		15	0/0	—	0.7	>0.05
	10	5.3–27.0	Twice daily for 9 days, starting on day −1	15	50/20	>0.3	0.1	>0.05
	32	5.3–27.0		15	10/0	>0.5	0.0	—
	10	5.0–40.0	Once only 4 hr post	26	50/25	>0.3	0.2	>0.05
	32	5.0–40.0		26	0/0	—	0.6	>0.05
	10 (ip)	21.0–30.0		30	0/10	—	0.0	—
	32 (ip)	21.0–30.0		30	0/20	—	0.5	>0.05
HS	10	5.0–27.0	Once daily 1 through 7 days	15	50/30	>0.3	0.0	—
	32	5.0–27.0		15	30/10	>0.3	0.1	>0.05
	10	5.3–27.0	Twice daily for 11 days, starting on day −1	15	40/40	—	0.0	—
	32	5.3–27.0		15	20/20	—	0.0	—
Polio	10	0.6–5.0	Twice daily for 17 days, starting on day −1	21	0/0	—	0.0	—
	32	0.6–5.0		21	0/0	—	0.0	—
PR8	100 (in)	0.3–2.0	Once daily 1 through 9 days	21	0/10	—	2.0	<0.05
	320 (in)	0.3–2.0		21	10/0	>0.3	0.0	—
WR	10	1.5–12.0	Twice daily for 11 days, starting on day −1	27	10/10	—	0.8	>0.05
	32	1.5–12.0		27	0/5	—	0.0	—

[a] Unless otherwise indicated, virus was inoculated intracerebrally on day zero; ip = intraperitoneal; in = intranasal.

[b] Only nontoxic drug dosages considered (<30% mortality in drug controls at the end of the experiment).

[c] Probability that the observed increase in the per cent survivors in the drug-treated groups compared with the virus control group was due to chance. Determined by chi-square analysis.

[d] Probability that the observed increase in the mean survival of the drug-treated groups compared with the virus control groups was due to chance. Determined by *t* test.

sion of a tumor homograft rejection response. In these investigations, an L1210 tumor homograft rejection system in C57Bl mice was used. Trapani (*personal communication*) has shown that BCNU exerts no demonstrable suppressive effect upon development of anti-red blood cell antibodies. These investigations would imply that, if either BCNU or MTX is tolerance-inducing, their actions are, at least, different from that of X irradiation and the other alkylating agents tested.

Virtually negative results were obtained in the tests with BCNU against EEE, WEE, SF, HS, WR, PR8, RSV, and poliovirus infections in mice, although slight increases in life span occurred in EEE, SF, and PR8 virus-infected

TABLE 9. *Effect of 1,3-bis(2-chloroethyl)-1-nitrosourea upon friend leukemia virus (FLV)-induced splenomegaly in mice and Rous sarcoma virus (RSV)-induced tumors in chicks*

Virus	Drug dose (mg per kg per day)[a]	Test mice (survivors/ total)	Avg spleen or tumor wt (g)	Per cent of virus control spleen or tumor Wt	Spleen or tumor wt reduction P^b
FLV[c]	24.0	8/10	0.36	47	<0.05
	12.0	10/10	0.26	34	<0.05
	6.0	10/10	0.31	40	<0.05
	3.0	10/10	0.24	31	<0.05
	0	20/20	0.78		
RSV[d]	1.0	8/10	4.22	135	—
	0.5	9/10	3.17	101	—
	0.3	9/10	2.31	73	>0.05
	0.1	8/10	3.94	126	—
	0	8/10	3.13		
	10.0	7/10	1.61	70	>0.05
	5.0	10/10	1.20	52	>0.05
	2.5	9/10	1.58	69	>0.05
	1.3	10/10	1.60	70	>0.05
	0	10/10	2.28		

[a] FLV = treated ip twice daily for 9 days, starting on day −1. RSV = treated ip twice daily for 11 days, starting on day −1.

[d] Probability that the observed reduction in the average spleen or tumor weight compared with virus control animals was due to chance. Determined by *t* test.

[c] Inoculated ip into 18- to 21-g Swiss mice on day zero.

[b] Inoculated subcutaneously into the wing web of 40- to 60-g Heisdorf-Nelson chicks on day zero.

mice treated chronically with BCNU. In light of this possible activity, further tests are contemplated in the hope of finding an optimal time of treatment and drug dosage.

It was of interest that BCNU exhibited activity against FLV, since Chirigos (1964) reported this compound was effective in retarding splenomegaly and inhibiting virus in the plasma of animals infected with the related Rauscher leukemogenic virus. The average spleen weights of BCNU-treated animals infected with either FLV or Rauscher virus were, however, greater than the weight of normal mouse spleens under the same conditions.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service contract PH-43-63-68 from the National Cancer Institute, Cancer Chemotherapy National Service Center.

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