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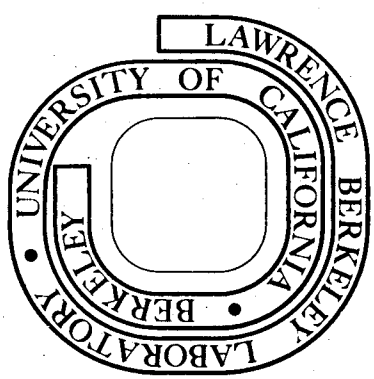

DISCLAIMER

Inhibition of Adenocarcinoma TA3 Ascites Tumor Growth by Rifamycin Derivatives[1]

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Running Title: Antitumor Activity of Rifamycin Derivatives

FOOTNOTES

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[3] The abbreviations used are: Rif, rifampicin; DMB, dimethylbenzyl-desmethylrifampicin; R-8_2, rifazone-8_2; MeC solution, 0.5% methylcellulose in 0.9% NaCl solution.

SUMMARY

A significant growth inhibitory effect on adenocarcinoma TA3 ascites tumors in LAF_1/J mice resulted from administration of 3 rifamycin derivatives: rifampicin (Rif), dimethylbenzyldesmethylrifampicin (DMB), and rifazone-8_2 (R-8_2). Drug injection was initiated on the first day after a 500 TA3 cell challenge, and a subtoxic 2 mg dose was given i.p. at 2 day intervals for a period of 3 or more weeks. Drug injections were made in 0.9% NaCl solution containing 0.5% methylcellulose. This high viscosity vehicle was found to be essential for obtaining a uniform drug suspension and a significant antitumor effect by the least water soluble derivatives, DMB and R-8_2. The more hydrophilic derivative, Rif, was found to have a comparable growth inhibitory effect on TA3 cells when prepared in 0.9% NaCl solution with or without added methylcellulose. A 3-week drug injection protocol resulted in mean survival times of 44.3, 34.0 and 46.7 days for tumor-bearing mice receiving Rif, DMB and R-8_2, respectively. In comparison, the mean survival time for control tumor-bearing mice that were injected i.p. with vehicle only was 18.9 days. In addition, while no survivors were ever observed in the control tumor-bearing animals, the administration of rifamycin derivatives, especially Rif and R-8_2, resulted in up to 50% tumor cures. With Rif and DMB, evidence was obtained that increasing the period of drug injection beyond 3 weeks does not lead to an increase in antitumor activity. The derivative R-8_2, when given orally as an oil-lecithin suspension, was found to have no antitumor effect.

INTRODUCTION

Rifamycin derivatives have been demonstrated in vitro to inhibit RNA-instructed DNA polymerase activity (6-9, 21, 23, 24, 27), and to prevent cellular transformation by RNA viruses (3, 4, 10, 11, 15, 17, 22). Rifamycin derivatives have also been shown in cell culture studies to be selectively toxic

to virally-transformed chick cells (2, 26) and leukemic leukocytes (16, 18), and to lead to the production of non-infectious progeny virus in transformed chick cells (19). In vivo experimental programs to evaluate the antitumor potential of rifamycin derivatives have been less extensive. Studies to date have demonstrated that Rif[3] inhibits Walker 256 ascites carcinosarcoma in rats (1) and adenovirus-induced tumors in hamsters (25). The derivative DMB has been observed to inhibit the onset and to decrease the total incidence of carcinogen-induced rat tumors (14). In this communication, we report a growth inhibitory effect of Rif, DMB and R-8_2 on rapidly-growing mouse adenocarcinoma TA3 ascites tumors, and discuss the importance of the drug vehicle and the route of administration.

MATERIALS AND METHODS

Chemicals. Rifamycin derivatives were obtained from the following sources: Rif (Calbiochem, Los Angeles, Calif.); DMB (Gruppo-Lepetite SpA, Milano, Italy); R-8_2 (synthesized in our laboratory (24) or obtained from Gruppo-Lepetite SpA). The structures of these 3 rifamycin derivatives are shown in Chart 1. Methylcellulose (4000 cp grade) was obtained from Matheson, Coleman and Bell (Norwood, Ohio). Vegetable lecithin was obtained from Nutritional Biochemicals Corporation (Cleveland, Ohio).

TA3 Ascites Tumors. The TA3 tumor used in these studies is a hypotetraploid subline of the TA3/Ha tumor originally isolated from an A/HeHa mouse in 1949 (12). The etiology of this tumor line has not been determined. Like the near-diploid TA3/Ha tumor, the hypotetraploid TA3 subline is widely allotransplantable and is only weakly immunogenic in syngeneic and isogeneic hosts (5, 13, 20). The TA3 tumor is maintained in our laboratory by weekly i.p. inoculation of 10^5 cells into isogeneic adult female LAF_1/J hosts (The Jackson Laboratory, Bar Harbor, Maine). During exponential growth phase, TA3 ascites

tumors in LAF_1/J hosts have a doubling time of 12 hr (20).

Transplantation of TA3 tumors for chemotherapeutic studies was carried out by the following procedure. Non-hemorrhagic ascites fluid was withdrawn from donor mice (using antiseptic conditions) and diluted 50 times with ice cold 0.9% NaCl solution. TA3 cells were counted in a hemocytometer, and a further dilution was made with ice cold 0.9% NaCl solution to give a final cell concentration of 5000 per ml. A 0.1 ml volume containing 500 cells was then injected i.p. into recipient female LAF_1/J mice. The cell suspension was maintained in an ice bath during transplantation, which was completed within 30 min following initial removal of the ascites fluid from donor mice. Viability of the transplanted TA3 cells was $>97\%$ based on exclusion of 0.5% nigrosin dye.

Drug Preparation. In most experiments where rifamycin derivatives were injected i.p., the drugs were prepared in a 0.9% NaCl solution containing 0.5% methylcellulose (hereafter denoted as MeC solution). The drugs were first ground with mortar and pestle while in the dry state. Approximately 2-3 ml of MeC solution was added and the grinding continued. The drug preparation was quantitatively transferred to a glass-walled teflon tissue grinder (A. H. Thomas, Philadelphia, Pa.) with an additional 20-25 ml of MeC solution, and the mixture homogenized at 600-700 r.p.m. for 2 min. The volume of MeC solution was then adjusted to make the final drug concentration equal to 10 mg/ml. In experimental procedures where rifamycin derivatives were injected in 0.9% NaCl solution without added MeC, the same preparative procedure was used.

In preparing $R\text{-}8_2$ for oral administration, the solid compound was suspended in a Wesson oil - lecithin mixture and dispersed by sonication. The final lecithin concentration was 5%, and the $R\text{-}8_2$ concentration was 30 mg/ml.

Drug Injection. Rifamycin derivatives were injected i.p. in a 0.2 ml volume every 2 days. The dosage used with all 3 derivatives was 2 mg per

injection (approximately 80 mg/kg per 2 day interval). No drug toxicity was noted at this dose level of Rif, DMB or R-8_2. The toxicity and chemotherapeutic effectiveness of higher dose levels are currently being investigated in our laboratory.

The derivative R-8_2 was given p.o. as 1 drop of the oil-lecithin mixture per day (approximately 1 mg per dose). The drug dosage was thus comparable to the dose level used in the i.p. injection protocol described above. No drug toxicity was noted in mice receiving R-8_2 orally.

RESULTS

Injection Vehicles. Two of the rifamycin derivatives, DMB and R-8_2, tested for a chemotherapeutic effect have limited water solubility. Therefore, a high viscosity 0.5% MeC solution (25 cp at 25°, 19 cp at 37°) was used to achieve a uniform drug suspension. As demonstrated by control TA3 tumor development following repeated i.p. injections of 0.9% NaCl solution with and without added 0.5% MeC (Tables 1 and 2), no inhibitory effect on tumor growth could be attributed to the MeC per se. Microscopic examination was made of Wright-Giemsa stained smears of ascites fluid from TA3 tumor-bearing mice receiving 6 i.p. injections of MeC solution. More than 98% of the cells were histologically distinguishable as TA3 cells. No evidence was obtained for MeC eliciting the appearance of inflammatory cells following repeated injection into the peritoneal cavity.

Rifampicin. Rif exerted a strong inhibitory effect on TA3 ascites tumor development, as shown in Chart 2. The mean survival time of tumor-bearing mice was increased 2.6- and 2.4-fold following i.p. administration of Rif for 21 and 36 days, respectively (Table 1). The difference in mean survival times observed with these two Rif injection protocols was not statistically significant (Table 2). Rif antitumor activity was found to be the same with

and without the use of MeC solution as a suspending vehicle (Chart 2, Tables 1 and 2).

DMB. As summarized in Chart 3 and Table 1, DMB inhibited the growth of TA3 tumors and increased the mean survival time of tumor-bearing mice by 1.7-, 2.2- and 1.9-fold when the drug was injected i.p. in MeC solution for periods of 21, 36 and 49 days, respectively. Although the greatest increase in survival time was obtained with a 36-day injection period, a statistical comparison of results for 21-, 36- and 49-day protocols suggests that DMB antitumor activity is not enhanced by extending the duration of treatment beyond 21 days (Table 2). In direct contrast to Rif, the use of MeC solution as a suspending vehicle was found to be essential for achieving a growth inhibitory effect by DMB (Chart 3, Tables 1 and 2).

Rifazone-8_2. The derivative R-8_2 was also found effective as an inhibitor of TA3 ascites tumor development when given i.p. in MeC solution (Chart 4). Administration of R-8_2 led to a 2.5-fold increase in the mean survival time of tumor-bearing mice relative to controls (Table 1). Attempts to inject R-8_2 in 0.9% NaCl solution without added MeC were unsuccessful because the drug could not be suspended adequately to allow syringe injection of a precise dosage.

In this series of experiments, i.p. injections of R-8_2 were not extended beyond 21 days; however, the effects of drug dosage, injection vehicle and duration of treatment on the antitumor activity of R-8_2 are currently being investigated, and will be reported in a later communication.

Antitumor activity of R-8_2 against TA3 ascites tumors was also examined following oral administration in an oil-lecithin vehicle. As shown in Chart 4 and Table 1, R-8_2 was found to have no growth inhibitory effect on TA3 tumors when given orally.

Tumor Cures. The number of tumor cures was recorded for both control and drug-injected mice. As shown in Table 1, all of the control mice used in these

experiments had expired by the 60th day following tumor transplantation. However, regardless of the mode by which the drugs were administered, survivors that were free of ascites tumors were observed at 60 days after treatment with rifamycin derivatives. The highest level of tumor cures, 30 to 50%, occurred following i.p. administration of Rif and R-8_2 (Table 1).

DISCUSSION

All 3 rifamycin derivatives tested for a chemotherapeutic effect were observed to inhibit significantly the growth of TA3 ascites tumors. Our experimental evidence indicates, however, that the efficacy of these compounds depends strongly upon the choice of injection vehicle and the route of administration. With regard to vehicle, it was found that suspension in a high viscosity MeC solution was essential for achieving a growth inhibitory effect by the poorly water soluble derivative DMB, but was not important for the activity of the more polar Rif derivative. The derivative R-8_2 is almost completely insoluble in water, and injection of this compound could be made only when MeC solution was used as a suspending vehicle. The effect of solution viscosity on DMB antitumor activity may be attributable to the maintenance of a uniform drug concentration within the peritoneal cavity, i.e. within the entire region of the developing TA3 ascites tumor. Also, the high viscosity suspending medium may reduce the rate of drug loss from the peritoneum into the general circulation. Regardless of whether one or both of these mechanisms is operative, the importance of the suspending vehicle appears to be related to the achievement of a sustained high concentration of drug in the tumor region. Consistent with this observation was the finding that no antitumor effect resulted from oral administration of R-8_2, whereas a strong growth inhibitory effect on TA3 ascites tumors was observed following i.p. injection. In order to more fully explain the role of the injection vehicle,

studies are currently being carried out to determine the rate of radioisotopic drug clearance with various suspending media. Also, additional chemotherapy trials with R-8_2 are being performed in order to evaluate more completely the dependence of antitumor activity on the route of drug administration, the injected dose level, the frequency of drug injection, and the overall length of the treatment schedule.

From a comparison of results obtained with Rif, DMB and R-8_2 suspended in MeC solution and injected at the same dose level, there appears to be no direct correlation between the antitumor activity of these compounds and their water solubility. The most hydrophobic derivative, R-8_2, and the least hydrophobic derivative, Rif, exerted nearly identical growth inhibitory effects against TA3 ascites tumors (Table 2). The derivative DMB, which has an intermediate water solubility, was found to be slightly less effective than either Rif or R-8_2 (Table 2). In analyzing the possible relationships between the water solubility of rifamycin derivatives and their <u>in vivo</u> activity against the TA3 ascites tumor system, three factors must be taken into consideration: (a) At the same injected dose level, the difference in water solubility of the various rifamycin derivatives may lead to significant differences in their effective concentrations within the peritoneal cavity. (b) The rates of clearance of the various rifamycin derivatives from the peritoneal cavity into the general circulation may differ. (c) Differences in lipophilicity of rifamycin derivatives may influence their relative penetration into TA3 cells. Kinetic studies with radioisotopically labeled rifamycin derivatives should be of value in determining the importance of each of these factors.

Initial interest in the chemotherapeutic potential of rifamycin derivatives resulted from the demonstration that these compounds serve <u>in vitro</u> as potent inhibitors of the RNA-instructed DNA polymerase (reverse transcriptase) enzyme system (6-9, 21, 23, 24, 27). The inhibitory effect of various rifamycin

derivatives on reverse transcriptase was shown to be closely correlated with their ability to prevent the transformation of cultured cells by RNA oncoviruses (3, 22). Later studies demonstrated that rifamycin derivatives can exert a selective growth inhibitory effect on RNA virus transformed cells, under conditions where the transformation is stable and no longer dependent on the presence of reverse transcriptase activity (2, 26). Finally, the derivative R-8_2 has been shown to inhibit the infectivity of Rous sarcoma virus by a mechanism that appears to be distinct from the inhibitory action of this compound on reverse transcriptase or its killing action on transformed cells (19). On the basis of these in vitro and cell culture studies, rifamycin derivatives thus appear to have several distinct modes of action leading to the inhibition of cell transformation, a selective cytotoxic effect on transformed cells, and the removal of viral infectivity. Analysis of the mechanism(s) by which rifamycin derivatives exert their in vivo growth inhibitory effect on TA3 tumor cells is hindered by a lack of precise knowledge regarding the etiology of this tumor line. Preliminary results obtained in our laboratory, however, indicate that TA3 cells do not possess detectable levels of reverse transcriptase activity (and thus RNA virus particles). It is conceivable that the mechanism of drug action in vivo may be either identical to, or closely related to, the selective cytotoxic action exerted against transformed cells in culture (2, 26). Investigations on the subcellular localization of radioisotopically labeled rifamycin derivatives, as well as the determination of their inhibitory effect on polymerases and possibly other cellular enzyme systems involved in nucleic acid and protein synthesis, should aid in elucidating the mechanism of antitumor activity. These experiments are currently in progress.

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Table 1. Effect of rifamycin derivatives on survival of LAF_1/J mice bearing adenocarcinoma TA3 ascites tumors

Injected compound and route[a]	Duration of treatment (days)	Number of mice	Number of cures[b]	Mean survival time ± 1 S.D.[c]
i.p. MeC	21	110	0	18.9 ± 4.8
i.p. 0.9% NaCl	21	20	0	19.0 ± 4.0
i.p. Rif in MeC	21	10	2	44.3 ± 13.8
i.p. MeC control	21	10	0	17.1 ± 4.3
i.p. Rif in MeC	36	40	20	51.4 ± 11.5
i.p. MeC control	36[d]	40	0	21.7 ± 4.5
i.p. Rif in 0.9% NaCl	21	20	7	51.4 ± 9.5
i.p. 0.9% NaCl control	21	20	0	19.0 ± 4.0
i.p. DMB in MeC	21	40	2	34.0 ± 13.9
i.p. MeC control	21	40	0	19.9 ± 4.1
i.p. DMB in MeC	36	20	6	47.0 ± 14.2
i.p. MeC control	36[d]	20	0	20.9 ± 3.4
i.p. DMB in MeC	49	20	2	39.2 ± 13.8
i.p. MeC control	49[d]	20	0	20.9 ± 3.4
i.p DMB in 0.9% NaCl	21	30	1	22.9 ± 11.6
i.p. 0.9% NaCl control	21	30	0	19.0 ± 4.0
i.p. $R\text{-}8_2$ in MeC	21	70	23	46.7 ± 12.5
i.p. MeC control	21	70	0	18.6 ± 3.7
p.o. $R\text{-}8_2$ in oil-lecithin	21	50	2	19.1 ± 11.3
untreated control	--	50	0	17.9 ± 4.6

Table 1. (continued)

(a) LAF_1/J mice were challenged with 500 TA3 ascites cells on Day 0, and drug treatment was initiated on Day 1. All rifamycin derivatives were administered i.p. as a 2 mg dose at 2-day intervals. Approximately 1 mg of $R\text{-}8_2$ was given p.o. every day.

(b) Mice surviving on Day 60 after tumor transplantation were considered to be cured. Tumor development subsequent to that time did not occur in any of the drug-treated mice.

(c) Mice that were cured were arbitrarily assigned a mean survival time of 60 days.

(d) Control mice were administered MeC solution i.p. until moribund.

Table 2. Statistical comparison of antitumor activities observed with different rifamycin derivatives and injection protocols

Comparison groups	Treatment procedures being compared[a]	p value[b]
MeC and 0.9% NaCl controls	21-day i.p. 0.9% NaCl (MST=19.0) vs i.p. MeC (MST=18.9)	N.S.
Drugs and MeC controls	21-day i.p. Rif in MeC (MST=44.3) vs i.p. MeC (MST=17.1)	<0.001
	36-day i.p. Rif in MeC (MST=51.4) vs i.p. MeC (MST=21.7)	<0.001
	21-day i.p. DMB in MeC (MST=34.0) vs i.p. MeC (MST=19.9)	<0.001
	36-day i.p. DMB in MeC (MST=47.0) vs i.p. MeC (MST=20.9)	<0.001
	49-day i.p. DMB in MeC (MST=39.2) vs i.p. MeC (MST=20.9)	<0.001
	21-day i.p. R-8_2 in MeC (MST=46.7) vs i.p. MeC (MST=18.6)	<0.001
Comparison of drugs in MeC	21-day i.p. R-8_2 in MeC (MST=46.7) vs 21-day i.p. Rif in MeC (MST=44.3)	N.S.
	21-day i.p. Rif in MeC (MST=44.3) vs 21-day i.p. DMB in MeC (MST=34.0)	<0.04
	21-day i.p. R-8_2 in MeC (MST=46.7) vs 21-day i.p. DMB in MeC (MST=34.6)	<0.001
Comparison of 21-, 36- and 49-day drug injections	36-day i.p. Rif in MeC (MST=51.4) vs 21-day i.p. Rif in MeC (MST=44.3)	N.S.
	36-day i.p. DMB in MeC (MST=47.0) vs 21-day i.p. DMB in MeC (MST=34.0)	<0.001
	49-day i.p. DMB in MeC (MST=39.2) vs 21-day i.p. DMB in MeC (MST=34.0)	N.S.
	36-day i.p. DMB in MeC (MST=47.0) vs 49-day i.p. DMB in MeC (MST=39.2)	N.S.
Drugs and 0.9% NaCl controls	21-day i.p. Rif in 0.9% NaCl (MST=51.4) vs 0.9% NaCl (MST=19.0)	<0.001
	21-day i.p. DMB in 0.9% NaCl (MST=22.9) vs 0.9% NaCl (MST=19.0)	N.S.
Comparison of drugs in 0.9% NaCl	21-day i.p. Rif in 0.9% NaCl (MST=51.4) vs 21-day i.p. DMB in 0.9% NaCl (MST=22.9)	<0.001

Table 2. (continued)

Comparison groups	Treatment procedures being compared[a]	p value[b]
Drugs in MeC and in 0.9% NaCl	21-day i.p. Rif in 0.9% NaCl (MST=51.4) vs 21-day i.p. Rif in MeC (MST=44.3)	N.S.
	21-day i.p. DMB in MeC (MST=34.0) vs 21-day i.p. DMB in 0.9% NaCl (MST=22.9)	0.001
Comparison of p.o. and i.p. route	21-day p.o. R-8_2 in oil-lecithin (MST=19.1) vs untreated controls (MST=17.9)	N.S.
	21-day i.p. R-8_2 in MeC (MST=46.7) vs 21-day p.o. R-8_2 in oil-lecithin (MST=19.1)	0.001

(a) The notations 21-day, 36-day and 49-day refer to the duration of drug treatment, beginning the first day after transplantation of 500 TA3 ascites cells. All compounds were administered i.p. as a 2 mg dose at 2-day intervals. Approximately 1 mg of R-8_2 was given p.o. every day. MST is the mean survival time of mice in a given treatment group; the number of mice per group is the same as that recorded in Table 1.

(b) p values were determined from a Student's "t" test for the significance of the difference in mean survival times. Values of p greater than 0.05 are denoted N.S. (not significant).

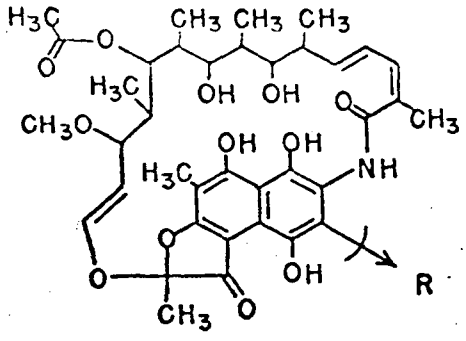
LEGENDS FOR CHARTS

Chart 1. Chemical structures of Rif, DMB and R-8_2.

Chart 2. The effect of Rif on the percentage survival of TA3 tumor-bearing mice is shown as a function of days following tumor inoculation. Two mg injections of Rif were given i.p. at 2-day intervals, beginning on the first day after tumor transplantation. Control mice received i.p. injections of either MeC solution or 0.9% NaCl solution on the same schedule. The number of mice in each treatment group is shown in parentheses.

Chart 3. The effect of DMB on the percentage survival of TA3 tumor-bearing mice is shown as a function of days following tumor inoculation. Two mg injections of DMB were given i.p. at 2-day intervals, beginning on the first day after tumor transplantation.

Chart 4. The effect of R-8_2 on the percentage survival of TA3 tumor-bearing mice is shown as a function of days following tumor inoculation. Panel A: Two mg injections of R-8_2 were given i.p. at 2-day intervals, beginning on the first day after tumor transplantation. Panel B: One mg doses of R-8_2 were given p.o. every day, beginning on the first day after tumor transplantation.



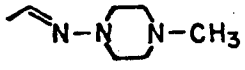
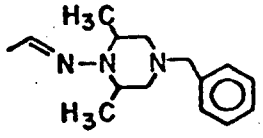
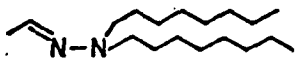
Compound	R
Rifampicin (Rif)	
Dimethylbenzyldesmethylrifampicin (DMB)	
Rifazone-8_2 (R-8_2)	

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Chart 1

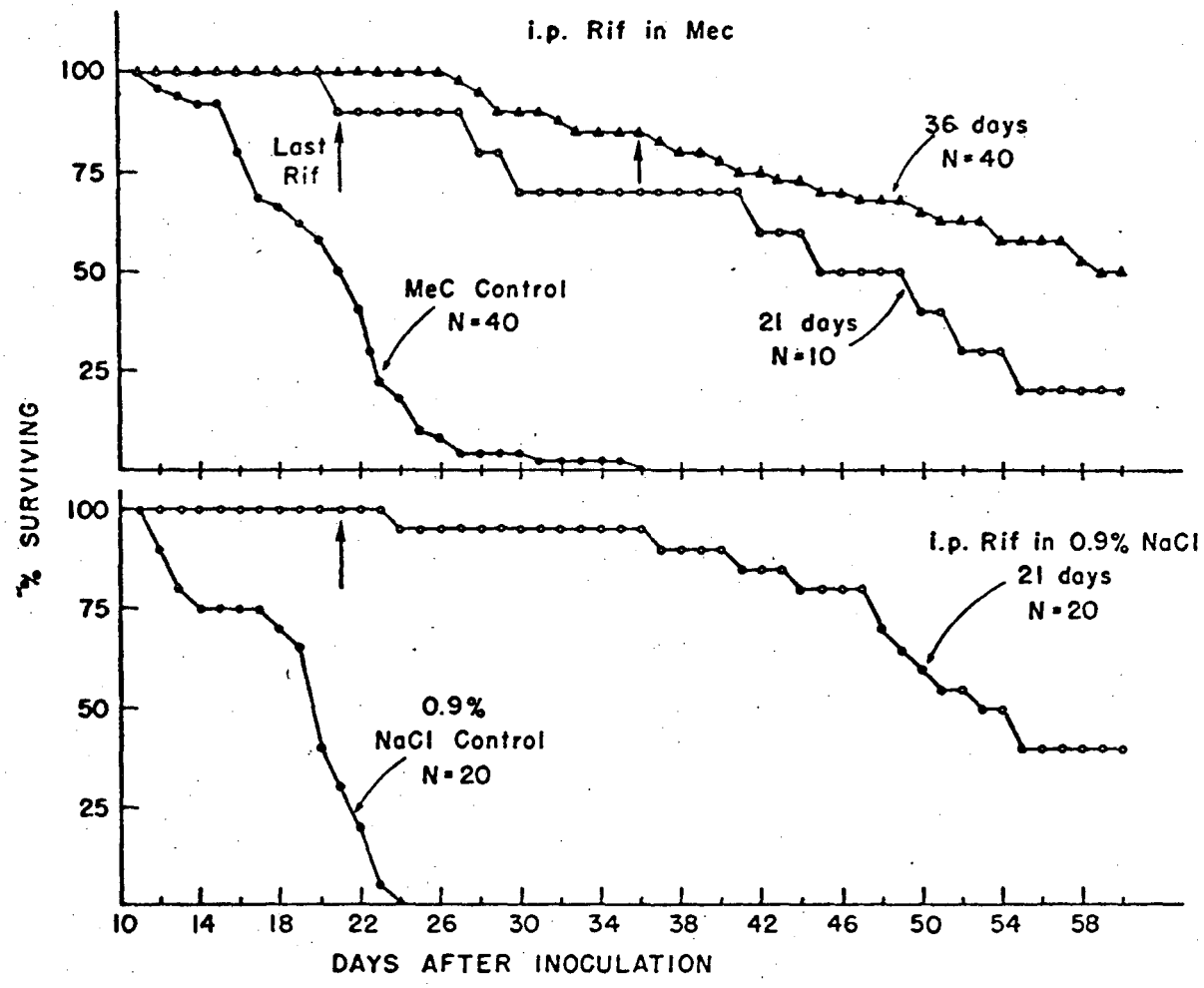

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Chart 2

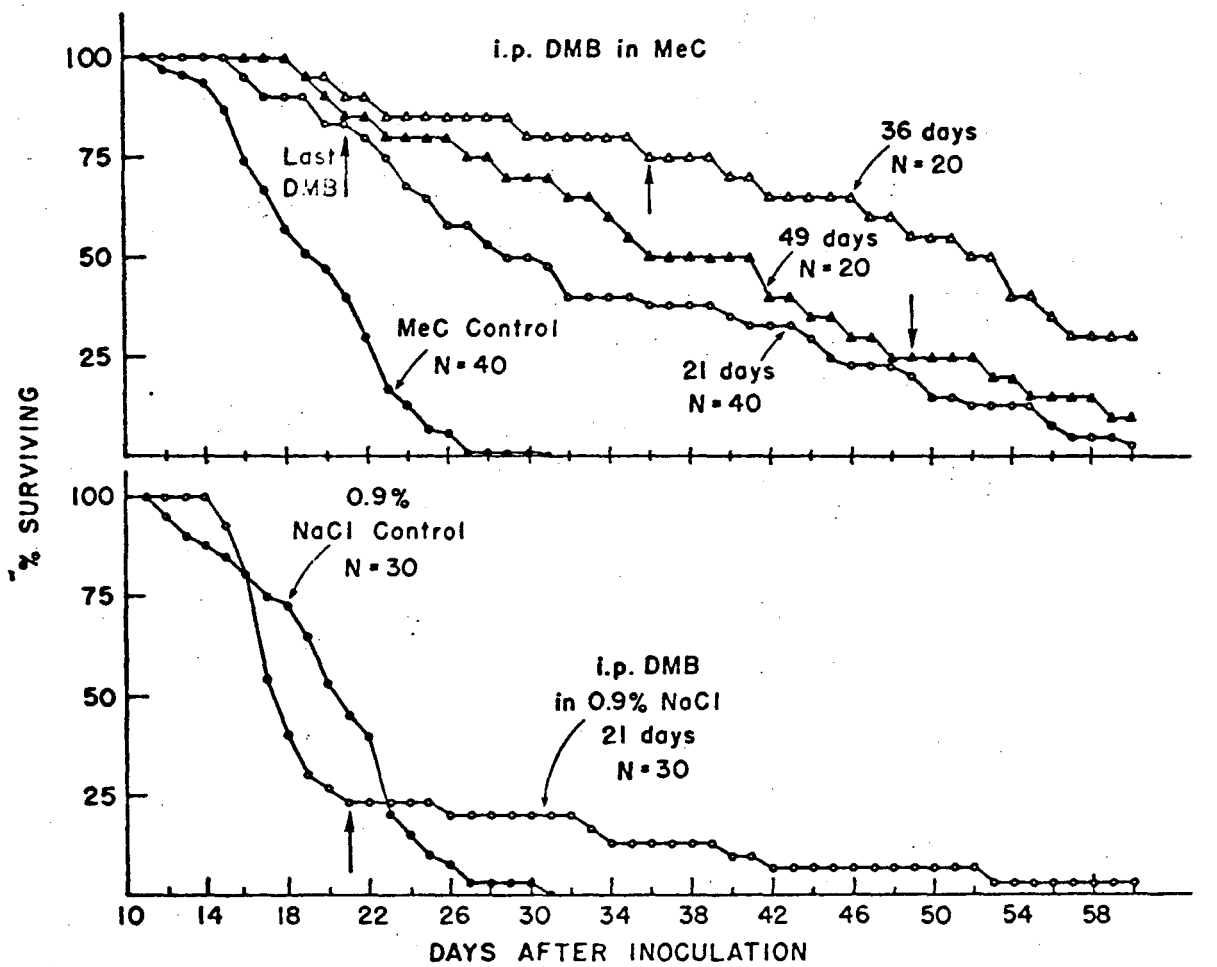
i.p. DMB in MeC
100
75
50
25
Last DMB
36 days
N = 20
49 days
N = 20
MeC Control
N = 40
21 days
N = 40
% SURVIVING
100
75
50
25
0.9%
NaCl Control
N = 30
i.p. DMB
in 0.9% NaCl
21 days
N = 30
10
14
18
22
26
30
34
38
42
46
50
54
58
DAYS AFTER INOCULATION
XBL 764-5826

Chart 3

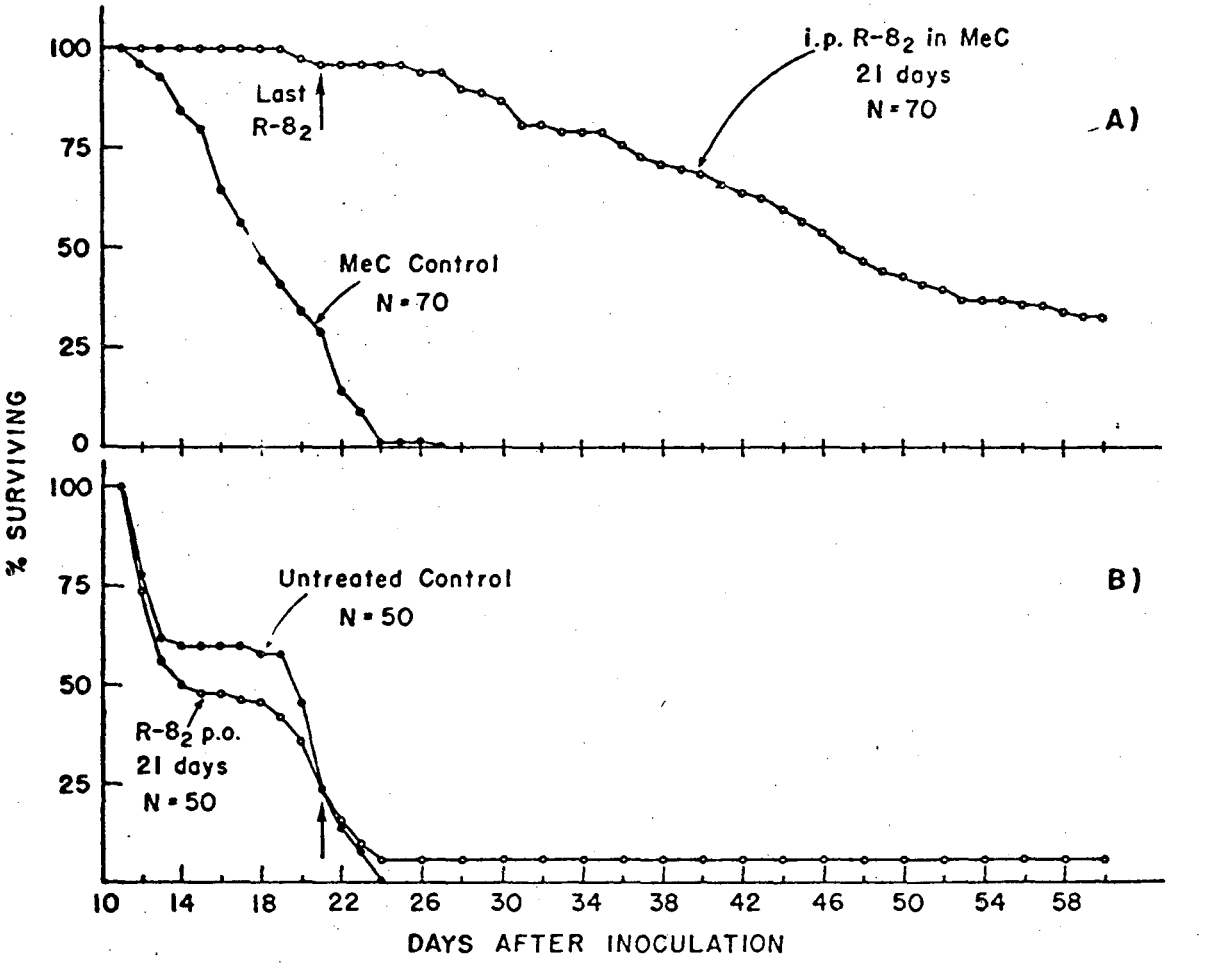

Chart 4

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