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ENHANCEMENT OF ANTIVIRAL AGENTS THROUGH THE USE OF CONTROLLED-RELEASE TECHNOLOGY

Annual Report

Thomas R. Tice, Richard M. Gilley, William M. Shannon,
and Melinda G. Hollingshead

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SOUTHERN RESEARCH INSTITUTE
2000 Ninth Avenue South
P.O. Box 55305
Birmingham, Alabama 35255-5305

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FOREWORD

In conducting the research described in this report, the investigators and their collaborators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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ENHANCEMENT OF ANTIVIRAL AGENTS THROUGH THE USE OF CONTROLLED-RELEASE TECHNOLOGY

I. SUMMARY

This is our Second Annual Progress Report on Contract DAMD17-86-C-6044, Southern Research Institute Project 5948: it covers research performed from February 1, 1987, to January 31, 1988. The major objectives of this research program are:

(a) To develop a programmed-release delivery system (microcapsule system) designed to enhance the immunogenic potential of an inactivated (killed) Japanese Encephalitis (JE) virus vaccine, allowing for immunization against this viral agent and,

(b) To develop controlled-release microcapsule delivery systems that will enhance the effects of the following immune modulators and antiviral agents: muramyl tripeptide (MTP), interferon (IFN), and poly(riboinosinic acid-ribocytidylic acid) [poly(I•C)]. More specifically, we are involved in the development of biocompatible, biodegradable, controlled-release microcapsule formulations to release poly(I•C), interferon (IFN), and JE vaccine at controlled rates after a single intramuscular or intravenous administration. We also plan to develop microcapsule formulations that will target the release of MTP to macrophages, causing macrophage activation and subsequent nonimmune protection against active viral infections.

During this year of the research program, we performed immunization studies with a prototype JE microcapsule formulation. The results obtained to date indicate the anti-JE antibody is present in the animals 136 days after administration of the prototype microcapsule formulation. These results are comparable to those of animals receiving the regular vaccination regimen with unencapsulated vaccine. Moreover, CPE inhibition assays and plaque-reduction assays were performed on the serum samples from immunized animals. The results of these assays indicate that the titers of neutralizing anitbodies for animals receiving a single administration of the prototype microcapsule vaccine formulation are similar to the titers obtained with three doses of unencapsulated vaccine although the kinetics for obtaining high-antibody titers are currently slower for the microcapsule formulation.

We continued working toward developing a more efficacious poly(I•C) microcapsule formulation. More specifically, we began developing poly(I•C) microcapsules which would release poly(I•C) at a continuous rate over a 30-day period, affording extended protection against viral challenge. Previously, we were developing a microcapsule formulation which would release poly(I•C) for 7 to 14 days.

We also began development of a muramyl tripeptide microcapsule formulation. A promising formulation has been prepared and will be tested during the next year.

II. INTRODUCTION

In today's battlefield enviroment, the threat of the use of viral biological warfare (BW) agents to incapacitate troops is real and serious. To minimize this threat, the need exists for the development of safe and effective vaccines that would immunize soldiers against BW agents. Numerous viruses have been identified as potential BW agents or as significant natural-disease threats to deployed military forces. Current technology allows production of either killed or live-attenuated vaccines to protect at-risk personnel.

Whether to use live (attenuated) or killed (inactivated) vaccines to provide individuals immunity form potentially fatal viral infections has been debated for many years. Live vaccines generally have offered the advantages of low cost per dose, high antibody production, and long duration of immunity. Their major disadvantages have been the occasional appearance of mild symptoms of the disease and their possible reversion to virulence. On the other hand, killed vaccines pose minimal health threats to the recipient. These killed vaccines, however, are not generally as immunogenic as live vaccines and produce lower antibody titers than do live vaccines. Further, recent experimental evidence has shown that killed vaccines do not provide protection when challenge virus is given by the aerosol route.

The strides recently made in recombinant DNA technology have led to a number of novel approaches to vaccination. Among these new approaches are cloning the viral proteins responsible for antigenicity and synthesizing specific peptide sequences that will elicit an immune response. The major limitation of these vaccines of the future is their reduced immunogenicity as compared to live vaccines. Several different approaches have been investigated to enhance immunogenicity of these vaccines. Among the most promising of these approaches are conjugation of the peptides to carrier proteins and the use of potent immunogenic adjuvants.

Previous research has demonstrated that the co-administration of immunogenic adjuvants (immune modulators) with killed vaccines greatly potentiates their immunogenicity. The early adjuvants proved highly effective in stimulating antibody production. These adjuvants, however, sometimes produced granulomas or sterile abscesses after administration.

Over the past decade, advances in bacterial-cell-wall chemistry have led to the identification of specific cell-wall structural components of a number of bacteria that will potentiate immunogenicity without the side effects seen with earlier adjuvants. Two of the most widely studied components to date is muramyl dipeptide (MDP) and one of its synthetic derivatives, muramyl tripeptide-phosphatidylethanolamine (MTP). MDP is the minimal structural unit (492 daltons) with immunopotentiator activity that can replace Mycobacteria in Freund's complete adjuvant. And MDP has been found to enhance the humoral antibody response to antigens and vaccines when administered by the oral or parenteral route. More specifically, when administered with antigens, MDP induces a more rapid primary

response followed by a stronger and more prolonged secondary antibody response. The co-administration of MDP with vaccines shows great potential in safely and effectively immunizing soldiers against potential BW agents.

In addition to enhancing the humoral antibody response to vaccines, MDP will also initiate cell-mediated responses against active viral infections by activating macrophages. More specifically, MDP has been shown to increase the cytolytic activity of macrophages in vivo after intravenous administration.

To be effective (MTP is rapidly cleared from the bloodstream), it must be encapsulated with a protective membrane and release only after it has been ingested by the macrophage. Encapsulation of MDP inside liposomes has been shown to be highly effective in activating liver macrophages to treat various viral infections. The major disadvantage of liposome carriers, however, is that all of the MDP is released quickly after the liposome is phagocytized, generally within hours.

This quick release requires the use of multiple injections to ensure that macrophages remain activated long enough to effectively cytolize the infecting virus. Also, liposomes are not as flexible as carriers for drugs as are other systems, e.g., microcapsule systems. More specifically, problems exist in preparing liposomes so that they will remain stable and not leak the encapsulated agent when injected into the body. In addition, liposomes must be stored in an aqueous enviroment to remain stable, requiring bulky containers and containment of water. On the other hand, microcapsules can be stored as a free-flowing powder that need only to be placed into aqueous solution immediately prior to injection. Moreover, microencapsulation processes are less difficult to scale up than the liposome fabrication process, especially in light of the amount of material that would be required for extensive immunization. Microcapsules lastly have the advantage in that they would remain stable for extend periods and following administration can be designed to release the MDP at a controlled rate for one to two weeks after ingestion by the macrophage. This mechanism of MDP availability would be more desirable.

In light of the advantages of controlled-release microcapsule delivery systems, it is the scope of this research to develop microcapsule delivery systems for JE vaccine, and for the immune modulators and antiviral agents muramyl tripeptide, interferon, and poly(I•C).

III. PREPARATION AND CHARACTERIZATION OF POLY(I•C) MICROCAPSULES

During this year of the project, we continued to produce and characterize poly(I•C) microcapsules for in vivo efficacy testing at USAMRIID and for pharmacodynamics studies at Southern Research. Research conducted during the first year of the project with poly(I•C) microcapsules was primarily directed towards identifying the in vitro release profile that resulted in the most efficacious response to in vivo challenge with Rift Valley Fever (RVF) virus.

These studies indicated that the most efficacious response was obtained with Batch E100-020. These microcapsules released poly(I•C) at a controlled rate for about 11 days. Because Batch E100-020 showed promising results, we prepared additional batches of poly(I•C) microcapsules that had in vitro release profiles similar to this batch. These microcapsules were used for numerous in vivo studies both at USAMRIID and at Southern Research.

We also began developing poly(I•C) microcapsules that will release poly(I•C) for up to 30 days. Previously, we had developed formulations that released poly(I•C) for periods ranging from 1 to 14 days. These longer-acting microcapsules are designed to confer greater protection against the possible exposure to exotic viruses under military conditions. Although our first in vivo challenge experiments with these formulations were encouraging, additional work is needed to optimize the 30-day formulation.

We also continued our efforts to optimize the poly(I•C) microcapsule formulation. Much of this research was directed towards increasing the core loading of the poly(I•C) microcapsules so that the amount of microcapsules needed to administer an efficacious dose of poly(I•C) could be minimized. More specifically, we prepared microcapsules using both the sodium salt and physiological salt of poly(I•C). Moreover, these batches were prepared using different microencapsulation processes, polymers, and solvents.

After preparation, we thoroughly characterized each batch of microcapsules. This characterization included examination of the microcapsules' surface morphology by scanning electron microscopy (SEM), determination of the core loading [in units of wt % poly(I•C)], and assessment of the in vitro release kinetics for each batch. The procedures we used to characterize the poly(I•C) microcapsules are outlined in our First Annual Progress Report on Pages 11 and 12. Table 1 shows characterization results of each batch of poly(I•C) microcapsules prepared during this reporting period. And Figures 1 and 2 show photomicrographs of typical poly(I•C) physiological salt and sodium salt microcapsules. The in vitro release profiles of each poly(I•C) microcapsule formulation tested in vivo are shown in Figures 3 and 4. In addition, the in vitro release profile of a prototype batch of poly(I•C) sodium salt microcapsules is illustrated in Figure 5.

IV. IN VIVO EXPERIMENTS WITH POLY (I•C)

A. Dose Response Study with Microencapsulated Poly(I•C)--Batch E100-020

Because our best survival rate (90%) during the past year was obtained after administration of 500 μg of microencapsulated poly(I•C) from Batch E100-020, we decided to conduct a dose-response study with this formulation to determine the minimal dose that would be required to confer protection against viral challenge. More specifically, additional

challenge experiments were conducted at USAMRIID using 250 and 100 μg of encapsulated poly(I•C) from Batch E100-020. In the survival experiments, the test mice were injected intraperitoneally (IP) with this poly(I•C) microcapsule formulation on Day -1 of the study. The mice were then injected subcutaneously (SC) with RVF virus (250 pfu) on Day 0. The mice receiving the microcapsules as well as untreated controls were then monitored for survival over the next 18 days.

The survival rates we obtained for the 500-, 250-, and 100-μg doses of microencapsulated poly(I•C) are shown in Figures 6 through 8. As can be seen, the results obtained with the two lower doses were disappointing. Although both of these doses extended the survival time of the test animals, most of the animals died after challenge. These data suggest that the amount of poly(I•C) being released from the microcapsules at these doses was insufficient to confer protection.

The in vitro release profiles we obtained for the three doses of microencapsulated poly(I•C) are also shown in Figures 6 through 8. In vitro, the 500-μg dose released about 40 to 60 μg of poly(I•C) per day, the 250-μg dose released about 20 to 30 μg per day, and the 100-μg dose released about 10 to 15 μg per day. It is important to note that the in vitro release rate is not intended to demonstrate a one-to-one correlation with the in vivo rate of release. (The release rate obtained in vivo can depend largely upon the route of administration which affects the cellular enviroment and hence the fluid perfusion kinetics to which the microcapsules will be exposed.) In vitro rates are important, however, for predicting how future batches of poly(I•C) microcapsules will release in vivo after base-line in vivo data have been obtained.

Survival rates obtained after multiple dosing with unencapsulated poly(I•C) (discussed in Section IV.C of this report) indicate that 20 μg of poly(I•C) per day will confer protection against viral challenge. These data suggest that poly(I•C) is being released from the microcapsules more slowly in vivo than in vitro because the 250-μg dose of encapsulated poly(I•C) which released 20 to 30 μg of poly(I•C) per day in vitro, did not protect the test animals.

In addition to the viral challenge experiments, we conducted pharmacodynamics studies on the 250- and 100-μg doses of encapsulated poly(I•C). The pharmacodynamics studies involved administering the microcapsules IP to animals and measuring interferon levels in the serum of the animals. The exact protocol we used for these studies is contained in Section V of this report.

The results of the pharmacodynamics studies are shown in Figures 9 through 11. As can be seen, significantly elevated serum interferon levels were only detected in the 24-h time interval following administration of the encapsulated poly(I•C). Moreover, there were no significant differences in the interferon levels measured for the animals receiving each dose (see Figure 12). Because there were significant differences in the survival rates obtained with animals treated with different doses of encapsulated poly(I•C), we feel that poly(I•C) might be conferring immunity by some mechanism other than interferon induction.

B. Comparison of the Subcutaneous and Intraperitoneal Routes of Poly(I•C) Microcapsule Administration on Interferon Induction

In addition to the pharmacodynamics studies conducted with various doses of microencapsulated poly(I•C), we also performed pharmacodynamics studies to determine if the route of administration would affect interferon serum titers obtained in mice treated with microencapsulated poly(I•C). More specifically, we administered the same dose of encapsulated poly(I•C) to test animals by the SC and IP routes and measured the daily serum interferon titers of these animals. Unlike all previous pharmacodynamics studies, the microcapsules used for this study (Batch E100-103) had been sterilized with 2.0 Mrad of gamma radiation. The exact protocol we used in this experiment is detailed in Section V of this report.

Our results indicated that administration of microencapsulated poly(I•C) by the SC versus the IP route resulted in significantly different serum interferon titers at the timepoints tested. Peak titers were present in the samples collected at Day 1 post-administration by both routes although the titers occurring after IP administration were as much as tenfold higher than those occurring after SC administration. Based on the 7 day titration curves, peak serum titers would have occurred between 4 and 48 hours post-injection by both routes of administration. However, titers returned to base line 2 to 3 days after IP administration, while increased titers were still detected 6 days after SC administration (Figure 13). Moreover, our results indicate that sterilization does not adversely affect the ability of microencapsulated poly(I•C) to induce interferon. This is in contrast to the results we obtained with sterilized, unencapsulated poly(I•C) (see Section D below).

C. In Vivo Experiments with Unencapsulated Poly(I•C)

In addition to the in vivo experiments conducted with microencapsulated poly(I•C), we also performed several experiments with unencapsulated poly(I•C) in an attempt to determine what dose and frequency of administration would result in protection against viral challenge. In these challenge experiments, mice were injected IP on Day -1 to 3 with either 5, 20, or 500 μg of unencapsulated poly(I•C). One additional group of mice received a single IP injection of 500 μg of unencapsulated poly (I•C) on Day -1. The mice were then exposed on Day 0 to 2500 pfu of RVF virus, and their survival was measured for 18 days.

The results of these survival studies are shown in Figures 14 through 16. Several important conclusions can be drawn from these studies. First, the animals treated with daily injections of 5 μg of unencapsulated poly(I•C) had poor survival rates, whereas all animals treated with daily injections of either 20 or 500 μg of unencapsulated poly(I•C) survived. These data indicate that the amount of poly(I•C) delivered is critical to ensure efficacy. Second, the animals treated with a single dose of 500 μg of unencapsulated poly(I•C) also had poor survival rates, indicating that controlled release of poly(I•C) will increase protection in animals against viral challenge.

Concurrent with the survival experiments, we also performed pharmacodynamics studies with unencapsulated poly(I•C). More specifically, we administered either 5 or 20 μg of unencapsulated poly(I•C) IP on Days 0 to 6 to test animals and measured their serum interferon titers. The results of these experiments are also shown in Figures 14 and 15. As can be seen, there are no significant elevations in interferon titers in the test groups over the titers of the untreated controls. This information is important because it indicates that the daily administration of poly(I•C) can result in efficacy against viral challenge without inducing the production of interferon. Obviously, some other mechanism of immunity is involved.

D. Effect of Gamma Radiation on Unencapsulated Poly(I•C)

We performed an experiment to determine whether gamma radiation would adversely affect the interferon-inducing ability of unencapsulated poly(I•C). Previously, we found that gamma radiation does not appear to affect the interferon-inducing capacity of encapsulated poly(I•C) (see Section B above). The protocol that we used for this experiment is detailed in Section V of this report. The mean serum interferon titers for 5 animals per group on Days -1 through 7 are presented in Table 2. The results indicate that gamma radiation adversely affects the interferon-inducing ability of unencapsulated poly(I•C).

E. In Vivo Challenge Study with Prototype 30-Day Poly(I•C) Microcapsules Batch E547-073

Toward the end of this reporting period, we prepared and characterized a microcapsule formulation designed to release poly(I•C) for 30 days in vivo. Prophylactic administration of poly(I•C) may be impossible, or at least impractical, immediately before the possible exposure to exotic viruses under military conditions. A long-term controlled-release microcapsule system for delivery of poly(I•C) would be advantageous as a means of administering the dose well before the time of exposure, or possible exposure to the virus. In previous work, we had developed microcapsule formulations that release poly(I•C) for time periods ranging from 1 to 14 days. To increase the period of protection, we have begun development of a microcapsule formulation which will release poly(I•C) at a continuous rate for up to 30 days.

To test the in vivo efficacy of this formulation, we prepared and sent 40 syringes containing 1000-μg doses of encapsulated poly(I•C) to USAMRIID for viral challenge experiments. Twenty of these syringes were administered SC 14 days before exposure to the challenge virus, and the other 20 were administered SC 7 days before the viral challenge. The in vitro release profiles for this formulation and the survival curves obtained after viral challenge are shown in Figures 17 and 18. As can be seen, the animals receiving the microcapsules at day -14 were not protected from the virus. On the other hand, animals receiving the microcapsules at Day -7 were afforded some protection.

Close examination of the in vitro release profiles for these doses show release kinetics that probably explain these results. For the animals receiving the microcapsules at Day-14, the amount of poly(I•C) being released at the time of viral challenge (Day 0) was very low (<10 μg per day). With the Day -7 animals, the release was somewhat higher (above 20 μg per day). Previous experiments have shown that at least 20 μg of poly(I•C) is needed per day to obtain efficacy. To improve this formulation, we plan to optimize the release profile of the microcapsules so that higher releases of poly(I•C) are obtained later on. This improvement in the formulation should result in greater efficacy against viral challenge.

F. Evaluation of Antiviral Efficacy of Unencapsulated Poly(I•C) Against Challenge with Venezuelan Equine Encephalomyelitis Virus

To determine the efficacy of poly(I•C) or other potential antiviral compounds, test animals must be exposed to viral challenge. The challenge virus should yield highly reproducible mortality rates in unprotected animals. Work has been conducted at USAMRIID with RVF virus and at Southern Research with JE virus. The mode of infecting test animals with JE virus (IP) has yielded erratic and unreproducible mortality rates, and thus an alternative viral model was sought. Venezuelan Equine Encephalomyelitis (VEE) virus was examined as a possible alternative. VEE virus is an alphavirus and is a member of the Togavirus family. VEE virus infections have been reported to be sensitive to the antiviral effects of interferon (1, 2). To determine whether this virus would be useful as a challenge model for poly(I•C), we administered various doses of unencapsulated poly(I•C) to animals and then challenged the animals with VEE virus. The protocol that we used for these experiments is described below.

The efficacy of poly(I•C) against VEE virus challenge in 4-week-old CD-1 (VAF+) female mice was assessed at each of three dose levels on 6 different treatment schedules. Mice received an LD_{90} challenge intraperitoneally on Day 0. Treatment groups consisting of groups of 10 infected and 5 uninfected mice received unencapsulated poly(I•C) at the doses and times listed below:

1. 500 μg of poly(I•C) on Day -4
2. 500 μg of poly(I•C) on Day -1
3. 100 μg of poly(I•C) on Day -1
4. 20 μg of poly(I•C) on Day -1
5. 500 μg of poly(I•C) on Days -1 through +4
6. 100 μg of poly(I•C) on Days -1 through +4
7. 20 μg of poly(I•C) on Days -1 through +4
8. 500 μg of poly(I•C) on Day +1
9. 500 μg of poly(I•C) on Day +2
10. 500 μg of poly(I•C) on Day +3
11. 100 μg of poly(I•C) on Day +1
12. 100 μg of poly(I•C) on Day +2
13. 100 μg of poly(I•C) on Day +3

Controls for this experiment included:

1.	Untreated, uninfected controls	(4 animals)
2.	Untreated, infected controls	(10 animals)
3.	Infected, PBS-treated day -4	(5 animals)
4.	Infected, PBS-treated day -1 through +4	(10 animals)
5.	Infected, PBS-treated day +1	(5 animals)
6.	Infected, PBS-treated day +2	(5 animals)
7.	Infected, PBS-treated day +3	(5 animals)

The mortality rates achieved in these experiments indicate that VEE virus is a viable model which can be used to assess the efficacy of poly(I•C) or other antiviral formulations in viral challenge experiments. Moreover, the results shown in Table 3 and illustrated in Figures 19 to 24, indicate that treatment of mice with as little as 20 μg of poly(I•C) on Days -1 through +4 provided significant protection against challenge with the Trinidad Donkey strain of VEE virus. In addition, significant protection was provided by a single dose of poly(I•C) administered on Day -1 prior to virus challenge.

V. PROTOCOLS FOR THE POLY(I•C) PHARMACODYNAMICS STUDIES

This section outlines the protocols that were used to perform the pharmacodynamics studies with unencapsulated and microencapsulated poly(I•C). During the course of evaluating the microencapsulated poly(I•C) formulations, several significant points became evident.

(1) The route of microcapsule administration has an effect on the maintenance of elevated serum interferon activity. Animals receiving microencapsulated poly(I•C) by the SC route maintained elevated serum interferon levels for a longer time interval than animals receiving similar microcapsules by the IP route. The variation seen in the two routes may be a result of differences in the rates of degradation of microcapsules or of the poly(I•C) at the two sites. Variations in the resident cell populations as well as variations in lymphatic and vascular drainage patterns may also play a role in the differences seen in the two routes of administration. Other workers (3) have reported a longer duration of serum interferon activity following injection of inactivated viral interferon inducers by the intramuscular (IM) route as compared to the IP or intravenous (IV) routes.

(2) Sterilization of unencapsulated poly(I•C) by gamma radiation may adversely affect its capacity to stimulate elevated serum interferon titers. Comparisons of serum interferon titers 4 h after administration of similar doses of irradiated and nonirradiated poly(I•C) indicate as much as a tenfold difference in serum interferon titers. However, it appears that microencapsulation of poly(I•C) protects it from degradation by gamma radiation. This finding is demonstrated by the high titers of serum interferon obtained with Batch E100-103, which was exposed to 2.0 Mrad of gamma radiation prior to administration to test animals.

(3) A four-hour interval from the time of poly(I•C) administration to the time of blood collection allows for detection of significant elevations in serum interferon titers. This is consistent with the kinetics of serum interferon titers reported by other workers (4, 5, 6).

(4) During interferon assays, pre-incubation of serum samples for 18 to 20 h prior to the addition of virus results in interferon titers that are three to four times higher than those measured in the 6-h pre-incubation assay. This increase in incubation time allows detection of smaller increases in interferon concentrations, thereby increasing the sensitivity of the assay.

A. Comparison of Two Doses of Microencapsulated Poly(I•C)

Virus-antibody-free CD-1 female mice were received at 3 weeks of age (10-12 grams) from Charles River Labs. After a 7-day quarantine period, the animals were randomly assigned to one of six experimental groups at the rate of 15 animals/group. Each group of 15 animals was further divided into 3 subgroups (5 animals each) designated A, B and C. The animals in each group were individually identified by ear notches. Blood was collected from one subgroup in each treatment group on Days -1 through 7 and then weekly on Days 14, 21, and 28. Blood collection was initiated 1 h post-treatment on the days when animals were treated. Blood samples were prepared and assayed for interferon titers using a vesticular stomatitis virus (VSV) inhibition assay as described in our First Annual Progress Report on Page 14. All treatments were administered IP. Treatment groups included:

(1) Untreated controls

(2) 5 μg of unencapsulated poly(I•C) administered daily on Days 0 through 6

(3) 20 μg of unencapsulated poly(I•C) administered daily on Days 0 through 6

(4) 100 μg of microencapsulated poly(I•C) administered on Day 0: Batch E100-020

(5) 250 μg of microencapsulated poly(I•C) administered on Day 0: Batch E100-020

(6) 500 μg of unencapsulated poly(I•C) administered on Day 0 only

Blood was not collected from Group 6 animals; they were toxicity controls.

The mean interferon titers for the 5 animals per group assayed each day are shown in Table 4. None of the Group 6 animals which received a single 500 μg dose of poly(I•C) died. Because of the increased interferon

levels seen in several of the untreated controls, representative animals from each group were assayed for antibodies to murine viruses using a commercial ELISA kit. All samples tested were negative for antibodies to mouse hepatitis virus, Sendai virus, Reovirus-3, and pneumonia virus of mouse.

The results of this experiment were disappointing in that significantly elevated serum interferon levels were only detected in the 24-h time interval following administration of microencapsulated poly(I•C). No elevations in serum interferon levels were detected in mice receiving 5 μg of unencapsulated poly(I•C) while mice receiving 20 μg of unencapsulated poly(I•C) had slightly elevated titers on Day 0. The lack of significantly elevated titers on Day 0 samples from these two groups is most likely the result of the timing of blood collection after poly(I•C) administration. A 1-h time interval from administration of inducer to collection of serum does not afford adequate time for serum interferon levels to increase significantly.

Slight increases were noted in Day 5 serum samples from animals receiving 20 μg of unencapsulated poly(I•C) and in those receiving 250 μg of encapsulated poly(I•C) when compared to the untreated controls; however, these increases were not statistically significant. Samples collected at time points beyond Day 5 are difficult to interpret because of increased titers present in the serum samples from untreated control animals.

B. Comparison of the Subcutaneous and Intraperitoneal Routes of Poly (I•C) Microcapsule Administration

Animals were randomly assigned to one of the three treatment groups. There were 15 animals per group, and each group was divided into 3 subgroups. One subgroup of each treatment group was bled daily on Days -1 through 7. Treatment groups consisted of

(1) Untreated controls

(2) 500 μg of microencapsulated poly(I•C) administered IP on Day 0: Batch E100-103-S

(3) 500 μg of microencapsulated poly(I•C) administered SC on Day 0: Batch E100-103-S.

Day-0 blood samples were collected 4 h after treatment.

The mean interferon titers of each group of 5 animals are shown in Table 5. These interferon assays were conducted as before except that the serum samples were pre-incubated with the cells for 20 h rather than 6 h prior to addition of virus.

Administration of the same dose of microencapsulated poly(I•C) by the SC route versus the IP route resulted in significantly different serum interferon titers. Titers peaked on Day 1 post-administration by both routes although the titers occurring after IP administration were as much

as tenfold higher than those occurring after SC administration. However, titers returned to base line 2 to 3 days after IP administration, while increased titers were still detected 6 days after SC administration.

The results of this experiment also indicate that a 4-h interval from the time of poly(I•C) administration to the time of blood collection is superior to a 1-h interval. This change in timing allows the detection of interferon synthesis occurring in the immediate post-treatment period.

The change in the interferon assays from a 6-h serum incubation to a 20-h serum incubation results in interferon titers which are three to four times higher; thus, this increases the sensitivity of the assay for detecting low levels of interferon activity.

C. Comparison of Gamma Irradiated and Non-irradiated Poly(I•C)

This work was conducted on the same format as the preceding experiment. Treatment groups included

(1) Untreated controls

(2) 50 μg of gamma-irradiated poly(I•C) IP on Days 0, 1, and 4

(3) 50 μg of non-irradiated poly(I•C) IP on Days 0, 1, and 4.

Blood was collected 4 h after treatment on days when treatments were administered. The mean interferon titers for 5 animals per group on Day -1 through Day 7 are shown in Table 2. These results indicate that gamma radiation adversely affects the interferon inducing ability of unencapsulated poly(I•C).

VI. PREPARATION AND CHARACTERIZATION OF JE VACCINE MICROCAPSULES

One of the major goals of this research program is to develop a programmed-release microcapsule system that with a single injection will afford a strong primary, secondary, and tertiary immune response to JE vaccine. More specifically, the final programmed-release system will consist of unencapsulated JE vaccine mixed with JE vaccine micro-encapsulated in two different DL-PLG excipients (50:50 DL-PLG and 65:35 to 85:15 DL-PLG). After administration, the unencapsulated JE vaccine will provide a primary immune response. Then, after the primary response has occurred, a 2- to 3-week period of dormancy should occur. After this dormancy period, the 50:50 DL-PLG excipient should begin to biodegrade and release the JE vaccine trapped within the microcapsule matrix, thereby eliciting the secondary response. After a second dormancy period, the microcapsules prepared with 65:35 to 85:15 DL-PLG will biodegrade and release the JE vaccine. This third pulsatile release will occur about 6 to 8 weeks after the microcapsules are administered and will elicit the tertiary response (see Figure 13). This programmed release of JE vaccine will allow complete immunization with a single injection.

During this year of research program, we prepared prototype JE vaccine microcapsules for initial in vivo immunization studies. These JE vaccine microcapsules were prepared using both 50:50 and 63:37 DL-PLG excipients. In addition, we continued our efforts toward optimization of the JE vaccine microcapsule formulation. More specifically, we began working with different microencapsulation solvents and techniques in order to improve the core loading and surface morphology of the JE vaccine microcapsules.

After preparation, we characterized each batch of JE vaccine microcapsules according to the procedures outlined in our First Annual Progress Report on Pages 16 and 18. The results of the characterization work show that we have improved the core loadings of the JE vaccine microcapsules while maintaining in vitro release profiles similar to those obtained with the microcapsules which were tested in the initial immunization studies (Batches E263-051 and E263-055). The higher core loadings will result in less material having to be administered to animals in order to achieve the same dose of vaccine. Figure 26 shows photomicrographs of typical JE vaccine microcapsules, and Table 6 lists the properties of the JE vaccine microcapsules prepared during this year.

VII. INITIAL IMMUNIZATION STUDY WITH THE PROTOTYPE JE VACCINE MICROCAPSULE SYSTEM

During this year, we performed an initial immunization study with our prototype JE vaccine microcapsule system. That is, we administered unencapsulated vaccine in conjunction with microencapsulated vaccine (prepared with both 50:50 and 63:37 DL-PLG excipient) to mice and measured the resulting antibody titers. The exact protocol we used to perform this immunization study is described below.

Seven-week-old virus-antibody-free CD-1 female mice (Charles River Labs) were randomly assigned to one of three treatment groups (Groups 1, 3 and 4) at 15 mice per group. Ten animals from each group were bled on a weekly schedule. The treatment groups included

(1) Untreated animals

(2) 0.2 mL (3.0 mg) of unencapsulated JE vaccine administered IP on Day 0

(3) 0.2 mL (3.0 mg) of unencapsulated JE vaccine administered IP on Days 0, 14, and 42

(4) 0.2 mL (3.0 mg) of unencapsulated JE vaccine, 1.5 mg of encapsulated JE vaccine prepared with 50:50 DL-PLG, and 1.5 mg of encapsulated JE vaccine prepared with 63:37 DL-PLG administered IP on Day 0.

Groups 1, 3 and 4 were on study simultaneously while Group 2 was part of a separate study. Day 0 was the same for animals in Groups 1, 3 and 4. All four groups of animals were sex, age, strain, and source matched. Also, the same Lot numbers of JE vaccine was used.

The results we have obtained to date from this immunization study are detailed in the following sections.

A. Results of ELISAs on Serum Samples from the Initial Immunization Study

In order to generate preliminary data while the CPE-inhibition and the plaque-reduction assays were being standardized, a JE-vaccine-based ELISA was used to evaluate serum samples from the immunized mice. The procedure that we used to perform the ELISAs follows.

Lyophilized JE vaccine is used as the antigen source. Lyophilized vaccine is weighed out and diluted in carbonate/bicarbonate coating buffer (pH 9.2) at a concentration of 0.25 mg/mL of solution. Next, 100 μL of the antigen solution is added to each well of 96-well microelisa plates, and the plates are incubated overnight at 4 °C for antigen coating.

After antigen coating, the plates are blocked with a 1% gelatin solution for 1 h at room temperature (RT). After blocking, the plates are washed once with phosphate-buffered saline (PBS, pH 7.2) containing 0.05% Tween 20 (PBS-TW). Serial twofold dilutions of sera to be analyzed are prepared in PBS-TW. Next, 50 μL of each dilution to be tested are added to duplicate or triplicate wells, and the plates are incubated 2 h at RT. The plates are then washed 3 times with PBS-TW and 50 μL of a 1:10,000 dilution of horseradish peroxidase. (HRP)-labeled goat anti-mouse IgG (prepared in PBS-TW) are added to each well. After 1 h of incubation at RT, the plates are washed 5 times with PBS-TW. After washing, 50 μL of substrate [ABTS/H_2 O_2] are added into each well, and the plates are incubated 30 min at RT. Plates are read in a microtiter plate reader with a 414-nm filter. The O.D. 414-nm readings are averaged for duplicate or triplicate samples. Positive and negative control sera are included on each plate.

To date, we have obtained ELISA results for this experiment through Day 136. These data are shown in Table 7. As can be seen, the results indicate that the antibody titers of the mice treated with the prototype microcapsule formulation are comparable to those of mice receiving 3 doses of unencapsulated JE vaccine on the regular immunization regimen.

To compare the results of the ELISAs run on serum samples collected at various time points during the experiment, we calculated the results of the 1:800 serum dilutions in the ELISA as a percent of the positive control serum which was included in each assay. By analyzing the data in this fashion we hoped to decrease the effects of day-to-day variations in the test so that the samples could be evaluated more easily. The 1:800 serum dilution was chosen because it most often represented the peak O.D. 414-nm values obtained for the serum samples. The ELISA results of the serum samples are presented in this fashion in Table 8.

The prototype JE vaccine microcapsule formulation is designed to release an additional quantity of the vaccine between 60 and 80 days post-injection. We have found that the Day 136 serum anti-JE antibody levels in mice treated with microencapsulated JE vaccine are comparable to mice treated with a normal schedule of unencapsulated vaccine. These results support the conclusion that the microencapsulated JE vaccine did release booster doses of anitgen which augmented the immune response to the primary anitgen exposure. Figure 27 illustrates the results of these studies.

B. Results of CPE Inhibition Assays on Serum Samples from the Initial Immunization Study

Because the results from the ELISA serum antibody titrations were so promising, we performed CPE-inhibition assays with the Day 21, 49, 77 serum samples to determine the levels of virus-specific neutralizing antibodies that were present. (Note: The results of the ELISA assay do not necessarily represent the amount of antibody that is specific to JE virus. Instead, it represents antibody titers against the rather impure JE vaccine.) The procedure that we used to perform the CPE assays follows.

Serum samples were heat inactivated at 56 °C for 30 minutes. Serial two-fold dilutions were prepared from the serum samples in Minimal Essential Medium supplemented with 5% heat-inactivated fetal calf serum starting with a 1:10 dilution. Each serum dilution (50 μL) was mixed with 50 μL of the virus preparation containing 100 $TCID_{50}$ of virus. The plates containing the virus/serum mixtures were incubated 1 h at 37 °C. Each serum dilution sample was assayed in triplicate. After incubation, 100 μL of medium containing 1.5 x 10_4 Vero cells were added to each test well. The plates were incubated at 37 °C in a 5% carbon dioxide atmosphere for 7 days. After incubation, the wells were fixed with buffered formalin for 1 h prior to staining with Coomassie Blue. CPE was read for each well (1+ = 25% CPE; 2+ = 50% CPE; 3+ = 75% CPE; 4+ = 100% CPE). Controls included (1) serum toxicity controls (serum dilution plus cells with no virus), (2) cell controls (cells plus media only), and (3) virus controls (cells plus virus with no serum). The serum titers were defined as that dilution of serum capable of reducing virus-induced CPE by 50% (2+).

The results of the CPE inhibition assays are presented in Table 9. Several conclusions can be extrapolated from these data. First, negative controls (Group 1 had no significant neutralizing antibody against JE virus, and a single 3.0-mg dose of unencapsulated JE vaccine (Group 2) administered IP does not induce high levels of neutralizing antibodies. Of the 10 animals assayed, one (10%) did not have neutralizing antibodies in the serum (animal A-1). The highest titer occurred occurred on Day 49 in Animal B-1, which had a tier of 254. The highest geometric mean titers occurred on Day 49 after which 7 of the 10 animals assayed had a drop in serum neutralizing antibody titers.

Second, animals receiving 3 doses of unencapsulated JE vaccine (Group 3) IP on Days 0, 14, and 42 had peak geometric mean titers on Day 49, which was one week after the third dose of vaccine was administered. Of these 10 animals, 8 had a drop in virus antibody titers after Day 49.

Third, animals receiving the prototype JE vaccine microcapsule formulation (Group 4) had the highest geometric mean titers on Day 77. Of the 10 animals assayed, 8 had increasing serum virus neutralizing titers from Day 21 to Day 77. Of the remaining 2 animals, 1 had the same titer on Day 49 and Day 77 while the other animal had a drop in titer from Day 49 to Day 77. The serum virus neutralization titers in animals receiving the prototype microcapsule formulation did not reach the high level achieved in animals receiving the 3 doses of unencapsulated vaccine by Day 77. However, the animals receiving the microencapsulated vaccine continued to have increases in serum antibody titers after Day 49, while the animals receiving the 3 doses of unencapsulated vaccine had a drop in antibody titer after Day 49.

These data suggest that the prototype microcapsule formulation will successfully immunize animals against JE virus. We believe that the delay seen with the microcapsule formulation in achieving high titers of serum virus neutralizing antibody in due to the pattern of release of the vaccine from the microcapsules. More specifically, we feel that the third pulse of JE vaccine intended to boost antibody titers has probably not occurred by Day 77. We intend to perform CPE inhibition assays on later time points to determine whether higher antibody titers are obtained. Moreover, in future formulations, we will incorporate a microcapsule component that will release the vaccine earlier, which should result in a faster elevation of serum antibody titers than is seen with the current formulation.

C. Results of Plaque Reduction Assays on Serum Samples from the Initial Immunization Study

We also shipped serum samples from the JE immunization study to USAMRIID so that plaque reduction assays could be performed. The results from pooled serum samples of various JE vaccine formulations are shown in Table 10 and in Figures 28 and 29. These results confirm the results seen in the CPE inhibition assays. More specifically, the results show that nonvaccinated animals had no neutralizing activity against JE virus (Nakayama strain) and that animals receiving a single 3.0-mg dose of unencapsulated JE vaccine developed low levels of neutralizing antibody activity. Moreover, animals receiving the standard three-dose regimen of 3.0 mg of unencapsulated JE vaccine IP on Days 0, 14, and 42 had peak serum titers on Day 49 which declined thereafter. On the other hand, animals receiving the prototype microcapsule formulation did not achieve high serum neutralizing antibody activity as rapidly as did the standard vaccine group: however, the microcapsule vaccine group continued to demonstrate increasing serum antibody titers after Day 49, at which time the standard vaccine group antibody activity had peaked.

These results indicate that single dose of the prototype microcapsule vaccine formulation results in antibody responses which are quantitatively similar to those present in animals receiving three doses of unencapsulated JE vaccine although the kinetics of increasing serum antibody titers are somewhat delayed in the prototype microcapsule vaccine group. To improve

our next JE microcapsule formulation, we plan to incorporate a faster-releasing component which should allow test animals to obtain high antibody titers more rapidly after the formulation is administered.

VIII. DEVELOPMENT OF A CHALLENGE MODEL FOR JE VIRUS

Initially, we attempted to develop a challenge model using the Nakayama strain of JE virus. However, this strain did not give reproducible mortality rates when mice were challenged by the IP route. Mortality rates were reproducible when challenge was administered intracranially (IC); however, IC challenge is difficult in 20-week-old animals and is not the challenge route of choice for evaluating anti-viral antibody activity.

In order to develop an in vivo JE virus model which uses IP or SC challenge rather that IC challenge, work with the Beijing strain of JE virus is in progress. More specifically, two vials of JE virus, Beijing strain, were received from Major Michael Ussery, USAMRIID. One vial contained JE virus which was prepared by infecting monolayers of Aedes albopictus C6/36 cells and the second vial contained JE virus which was prepared by infecting Vero cells. Upon receipt, half of the contents of each vial was inoculated into separate cultures of Vero cells to produce seed stocks. The seed stock derived from the C6/36 cell stock was used to prepare a working stock of virus in Vero cells. The working stock of Beijing strain JE virus derived from the C6/36 cell stock contains 8×10^6 pfu/mL of culture supernatant as determined by plaque titration in Vero cells.

The results of the initial in vivo titration through Day 21 post-challenge are presented in Table 11. Because of the lack of lethality of the virus challenge doses administered, another in vivo titration is currently in progress using challenge levels of 10,000 to 250 pfu per mouse. These challenge experiments are being conducted in groups of 12-week-old mice which are either infected and untreated or infected and subsequently treated with PBS by the SC route for 7 days to induce a stressor which may increase the host's susceptibility to JE virus challenge. In addition, mouse-brain-passaged virus is currently being prepared with the goal of enhancing the viral pathogenicity such that older mice may be susceptible to viral challenge.

IX. PREPARATION AND CHARACTERIZATION OF MURAMYL TRIPEPTIDE MICROCAPSULES

A. Preparation of Muramyl Tripeptide Microcapsules

During this year of research program, we successfully formulated a muramyl tripeptide (MTP) microcapsule formulation that warrants testing in vivo. More specifically, we prepared several batches of MTP microcap-

sules using varying encapsulation conditions. After preparation, each batch of microcapsules was thoroughly characterized using the procedures outlined below. Table 12 shows the results of the characterization studies, and Figure 30 shows photomicrographs taken by SEM of typical MTP microcapsules prepared during this reporting period.

After development of a promising formulation, we prepared a quantity of MTP microcapsules for in vivo efficacy testing. This batch of MTP microcapsules contains about 1.3 wt % MTP, and in vitro release analysis shows the MTP is being released at a controlled rate over about a 2-week period. We plan to test this formulation in vivo during the next year in conjunction with poly(I•C) and/or JE vaccine microcapsules.

B. Characterization of Muramyl Tripeptide Microcapsules

1. Core loading and encapsulation efficiency

The core loading of the MTP microcapsules is a measure of the amount of MTP incorporated inside the microcapsules. Our core loading procedure is based on the extraction of MTP core material from a known amount of microcapsules and quantification of the extracted MTP by a UV spectrophotometric assay. The procedure that we use follows.

A know weight of microcapsules is dissolved in methylene chloride. The MTP core material is then extracted into Nanopure water (Sybron-Barnstead, Boston, MA) which is then quantified for MTP spectrophoto-metrically at 205 nm. The theoretical core loading for a batch of microcapsules is based upon the copolymer and MTP input and is calculated in the following manner:

$$\text{Theoretical core loading, wt \%} = \frac{\text{MTP input, g}}{\text{(Copolymer input, g) + (MTP input, g)}} \times 100 \text{ wt \%}$$

The actual core loading is determined by assaying the microcapsules by the procedure described above. The actual core loading is calculated in the following manner:

$$\text{Actual core loading, wt \%} = \frac{\text{MTP assayed, g}}{\text{Microcapsules used in assay, g}} \times 100 \text{ wt \%}$$

The encapsulation efficiency is the ratio of the actual and theoretical core loadings and is calculated in the following manner:

$$\text{Encapsulation efficiency, \% of theoretical} = \frac{\text{Actual core loading, wt \%}}{\text{Theoretical core loading, wt \%}} \times 100 \text{ \%}$$

2. In vitro analysis

We determined the release kinetics of the MTP microcapsules using a short-term in vitro analysis. The purpose of this short-term in vitro analysis is to assess how well the MTP is encapsulated. If the micro-

capsules release a major amount of their MTP at an uncontrolled rate, they will most likely be ineffective in vivo.

The in vitro release analysis involves placing a known weight of microcapsules into a receiving fluid consisting of 0.15 M NaCl. This solution is placed in an incubator maintained at 37 °C. The receiving fluid is periodically removed and quantified for MTP spectrophotometrically. A fresh aliquot of receiving fluid is replaced into the in vitro apparatus.

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XI. ACKNOWLEDGMENTS

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Submitted by:

Melinda G. Hollingshead
Head, Viral Immunology and
Animal Models Section

Reviewed by:

William M. Shannon
Associate Director
Biochemistry Research and
Head, Virology Division

Richard M. Gilley
Head, Vaccine Delivery Section

Approved by:

Thomas R. Tice
Head, Controlled Release Division

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APPENDIX A

TABLES

TABLE 1. PROPERTIES OF POLY(I·C) MICROCAPSULES PREPARED WITH DL-PLG EXCIPIENTS

Microcapsule Batch	Polymer Batch	Core loading, wt % poly(I·C)		Encapsulation efficiency, % of	Poly(I·C) released in vitro, %, at				
		Theoretical	Actual	theoretical	1 h	3 h	4 h	24 h	48 h
E100-019	C779-090[a]	0.93	0.8	76.0	2.4	17.2	ND[b]	58.9	75.8
E100-020	C779-150[c]	0.49	0.3	51.0	0.8	2.7	ND	15.5	27.2
E100-103	C051-132[d]	0.47	0.4	85.8	1.0	2.9	ND	15.0	27.0
E100-103S	C051-132	0.47	0.3	72.3	4.5	ND	9.2	36.7	68.7
E100-137-1	C779-150	0.49	0.4	81.0	1.0	ND	2.7	14.1	25.3
E100-137-2	C779-150	0.48	0.3	62.0	0.9	ND	1.8	9.7	19.0
E100-139-2	C779-090	0.49	0.4	81.5	1.8	ND	4.7	15.0	26.4
E452-011	C051-132	0.48	0.4	83.0	1.5	ND	3.6	17.1	31.3
E452-061	C051-132	0.52	0.3	57.7	0.4	1.3	4.7	12.4	24.4
E452-075	D726-070[e]	0.50	0.4	80.0	1.9	4.8	8.7	25.1	38.3
E452-077	D726-070	0.52	0.5	96.2	1.7	6.7	8.8	24.1	33.6
E452-079	D726-070	0.51	0.6	117.6	2.8	8.1	13.0	30.4	40.2
E547-027	D466-027[f]	2.01	0.9	44.8	2.3	11.9	18.6	33.1	40.0
E547-029	D466-027	1.33	0.1	7.5	4.7	27.6	49.8	80.4	103.5
E547-073	D726-070	--	0.7	--	0.3	1.6	4.7	13.5	22.2
E547-073	C051-132	--	0.7	--	0.3	1.3	4.7	13.5	22.2
E547-105	D726-070	2.52	0.5	19.8	3.3	11.7	21.7	43.2	58.0
E547-109	D466-027	2.51	0.7	27.9	2.2	5.2	9.0	19.7	28.1
E547-135	D726-070	2.57	1.6	62.3	11.3	25.8	42.2	63.1	82.4
E547-141	D726-070	4.95	2.1	42.4	7.5	22.3	44.1	57.0	71.5
E547-147	D466-027	2.65	0.7	26.4	1.3	5.5	10.3	20.9	28.3
E598-035	D726-066[g]	2.41	2.1	87.1	0.5	0.9	1.5	2.3	3.1
E598-039	D466-027	1.44	1.0	69.4	2.9	7.8	11.3	18.6	32.4
E598-043	D726-066	2.55	1.5	58.8	1.2	2.0	2.9	4.7	6.5
E598-089	D726-066	2.53	1.8	71.1	1.2	2.1	2.8	3.4	4.1
E598-091	D726-066	3.46	2.5	72.3	0.5	1.1	1.5	2.2	2.8
E598-093	D466-027	1.53	0.3	19.6	1.6	3.2	5.2	14.3	26.6

[a] 54:46 poly(DL-lactide-co-glycolide), IV = 0.83 dL/g, HFIP, 30 °C, 0.5 g/dL.

[b] ND = Not determined.

[c] 66:34 poly(DL-lactide-co-glycolide), IV = 0.86 dL/g, HFIP, 30 °C, 0.5 g/dL.

[d] 63:37 poly(DL-lactide-co-glycolide), IV = 0.71 dL/g, HFIP, 30 °C, 0.5 g/dL.

[e] 65:35 poly(DL-lactide-co-glycolide), IV = 0.65 dL/g, HFIP, 30 °C, 0.5 g/dL.

[f] [illegible]-lactide-co-glycolide), IV = 1.20 dL/g, HFIP, 30 °C, 0.5 g/dL.

TABLE 2. EFFECT OF GAMMA RADIATION ON THE INTERFERON INDUCING CAPACITY OF POLY(I·C)

Group	Radiated poly(I·C)	Serum interferon titer on Day								
		-1	0	1	2	3	4	5	6	7
1	Control[a]	32	36	20	26	30	21	21	23	27
2	Yes[b]	24	345	391	56	49	197	21	24	22
3	No[c]	28	2384	469	81	44	2800	34	22	30

[a]Untreated controls.

[b]50 µg of gamma-irradiated poly(I·C) given IP on Days 0, 1, and 4.

[c]50 µg of poly(I·C) not irradiated, given IP on Days 0, 1, and 4.

TABLE 3. EVALUATION OF POLY(I·C) AGAINST VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS CHALLENGE

Treatment	Treatment given on Day	Number uninfected[a]	Number infected[b]	Day of death ± SD[c]
Untreated		0/4	6/10	7.7 ± 1.0
PBS	-4	ND[d]	4/5	9.3 ± 1.7
PBS	-1	ND	5/5	9.0 ± 1.2
PBS	-1 to +4	ND	10/10	8.3 ± 1.1
PBS	+1	ND	4/5	7.0 ± 0.8
PBS	+2	ND	5/5	7.8 ± 1.3
PBS	+3	ND	5/5	7.8 ± 0.8
500 µg poly(I·C)[e]	-4	ND	7/10	7.4 ± 3.4
500 µg poly(I·C)	-1	0/4	4/10	10.8 ± 1.7
100 µg poly(I·C)	-1	0/4	1/10	9.0 ± 0.0
20 µg poly(I·C)	-1	0/4	6/10	10.2 ± 4.1
500 µg poly(I·C)	-1 to +4	0/5	0/10	NA[f]
100 µg poly(I·C)	-1 to +4	0/5	1/10	8.0 ± 0.0
20 µg poly(I·C)	+1 to +4	0/5	3/10	14.3 ± 6.5
500 µg poly(I·C)	+1	ND	9/10	8.3 ± 1.9
500 µg poly(I·C)	+2	ND	8/10	8.5 ± 1.2
500 µg poly(I·C)	+3	ND	9/10	8.1 ± 2.6
100 µg poly(I·C)	+1	ND	9/10	8.4 ± 1.1
100 µg poly(I·C)	+2	ND	10/10	7.5 ± 1.4
100 µg poly(I·C)	+3	ND	10/10	7.5 ± 1.3

[a]Number of uninfected dead divided by the number of uninfected treated.

[b]Number of infected dead divided by the number of infected treated.

[c]Average day of death ± standard deviation.

[d]ND = Not determined.

[e]All poly(I·C) treatments consisted of poly(I·C) in aqueous 0.15 M NaCl administered SC on the indicated days. Animals were monitored for 21 days following virus challenge.

[f]NA = Not applicable.

TABLE 4. MEAN SERUM INTERFERON TITERS FOR MICE ADMINISTERED VARIOUS DOSES OF POLY(I•C)

Group	Serum interferon titer[a] at Day											
	-1	0	1	2	3	4	5	6	7	14	21	28
1[b]	≤21	≤22	≤20	≤22	≤22	≤20	≤21	≤33	42	≤24	≤53	≤21
2[c]	≤20	≤22	≤23	≤20	≤21	≤20	≤20	≤28	42	≤31	41	≤20
3[d]	≤28	≤38	≤21	≤24	≤23	≤23	≤36	≤34	≤35	≤37	≤32	≤28
4[e]	≤21	≤20	258	≤22	≤22	≤20	≤24	≤20	36	≤41	≤25	≤22
5[f]	≤24	≤20	382	≤21	≤26	≤21	≤47	≤21	≤33	≤50	≤24	≤20

[a] Interferon titer volume was 0.1 mL.

[b] Untreated controls.

[c] 5 μg poly(I•C) qld x 7.

[d] 20 μg poly(I•C) qld x 7.

[e] 100 μg encapsulated poly(I•C) administered on Day 0.

[f] 250 μg encapsulated poly(I•C) administered on Day 0.

TABLE 5. MEAN SERUM INTERFERON TITERS FOR MICE ADMINISTERED ENCAPSULATED POLY(I•C) BY VARIOUS ROUTES

Group	Serum interferon titer[a] at Day								
	-1	0	1	2	3	4	5	6	7
1[b]	≤24	≤20	≤20	≤20	≤22	≤27	≤22	≤21	≤25
2[c]	≤21	224	2309	50	≤24	≤22	≤25	≤23	≤20
3[d]	≤20	≤44	≥390	131	≤68	44	≤30	≤38	≤21

[a]Interferon titer volume was 0.1 ml.

[b]Untreated controls.

[c]Microencapsulated poly(I•C) administered IP.

[d]Microencapsulated poly(I•C) administered SC.

TABLE 6. PROPERTIES OF JE VACCINE MICROCAPSULES PREPARED WITH DL-PLG EXCIPIENTS

Microcapsule Batch	Polymer Batch	Core loading, wt %		Encapsulation efficiency, % of theoretical
		Theoretical	Actual	
E202-027	C799-150[a]	25.03	2.0	8.0
E202-041	C799-150	5.12	1.3	25.4
E202-065	C799-150	2.52	2.5	99.2
E202-071	C799-150	5.13	4.5	87.7
E202-073	C799-150	10.19	9.1	89.3
E202-081	C799-150	5.05	1.6	31.7
E202-085	C799-150	10.05	1.2	11.9
E202-127	D726-014[b]	5.08	2.8	55.2
E202-145	D726-014	5.09	1.2	23.6
E202-147	C799-154[c]	5.04	1.4	27.8
E263-051	D726-014	5.02	1.0	19.9
E263-055	C051-132[d]	5.01	1.0	20.0
E358-019	C799-022[e]	5.00	ND[f]	ND
E358-021	D726-014	5.11	ND	ND
E358-023	D743-007[g]	5.01	ND	ND
E358-037	D466-027[h]	5.03	1.0	19.9
E358-039	C779-124[i]	5.05	0.2	4.0
E358-041	D743-007	5.05	0.3	5.9
E358-043	C779-022	5.03	0.3	6.0
E358-047	D726-066[j]	5.00	0.6	12.0
E548-010	D466-027	5.62	3.1	55.2
E548-015	C779-154	5.60	1.2	21.4
E548-017	D726-070[k]	5.58	2.1	37.6
E548-023	D726-066	5.39	2.9	53.8
E548-025	C779-124	5.52	1.2	21.7
E548-043	D466-027	5.66	3.3	58.3
E548-045	C779-154	5.54	3.0	54.2

(continued)

TABLE 6 (continued)

Microcapsule Batch	Polymer Batch	Core loading, wt % Theoretical	Core loading, wt % Actual	Encapsulation efficiency, % of theoretical
E548-047	D726-070	5.49	1.5	27.3
E548-049	D726-066	5.61	1.4	25.0
E548-051	C779-124	5.29	1.2	22.7

[a]66:34 DL-PLG, IV = 0.86 dL/g, HFIP, 30 °C, 0.5 g/dL.

[b]50:50 DL-PLG, IV = 1.13 dL/g, HFIP, 30 °C, 0.5 g/dL.

[c]50:50 DL-PLG, IV - 0.63 dL/g, HFIP, 30 °C, 0.5 g/dL.

[d]63:37 DL-PLG, IV = 0.71 dL/g, HFIP, 30 °C, 0.5 g/dL.

[e]65:35 poly(DL-lactide-co-glycolide), IV = 0.65 dL/g, HFIP, 30 °C, 0.5 g/dL.

[f]ND = Not determined.

[g]50:50 poly(DL-lactide-co-glycolide), IV = 0.65 dL/g, HFIP, 30 °C, 0.5 g/dL.

[h]50:50 poly(DL-lactide-co-glycolide), IV = 1.20 dL/g, HFIP, 30 °C, 0.5 g/dL.

[i]50:50 poly(DL-lactide-co-glycolide), IV = 0.87 dL/g, HFIP, 30 °C, 0.5 g/dL.

[j]65:35 poly(DL-lactide-co-glycolide), IV = 0.94 dL/g, HFIP, 30 °C, 0.5 g/dL.

[k]65:35 poly(DL-lactide-co-glycolide), IV = 0.65 dL/g, HFIP, 30 °C, 0.5 g/dL.

TABLE 7. RESULTS OF ANTI-JE ANTIBODY TITRATIONS OF SERUM SAMPLES FROM THE JE VACCINE IMMUNIZATION STUDIES

Group	Number of animals assayed	Mean O.D. $_{414}$ readings of serum samples at serum dilutions of							
		1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600
Day 14									
3[a]	2	0.234	0.211	0.164	0.127	0.086	0.047	0.035	0.022
4[b]	2	0.208	0.163	0.123	0.072	0.051	0.033	0.026	0.025
Day 21									
1[c]	1	0.014	0.012	0.005	0	0.003	0.002	ND[d]	ND
3	5	0.251	0.293	0.3	0.282	0.247	0.199	0.129	0.076
4	5	0.279	0.267	0.249	0.184	0.139	0.079	0.042	0.025
Day 35									
1	5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	5	0.429	0.449	0.422	0.324	0.268	0.164	0.087	0.058
4	5	0.439	0.445	0.448	0.409	0.305	0.212	0.116	0.069
Day 49									
1	10	0.067	0.057	0.057	0.053	0.061	0.053	0.058	0.06
3	10	0.737	0.866	0.899	0.816	0.752	0.614	0.397	0.261
4	10	0.694	0.75	0.846	0.805	0.658	0.472	0.311	0.187
Day 63									
1	10	0.056	0.059	0.06	0.062	0.057	0.058	0.062	0.07
3	10	0.414	0.622	0.66	0.727	0.658	0.61	0.397	0.339
4	10	0.323	0.558	0.585	0.573	0.524	0.372	0.257	0.165
Day 77									
1	10	0.041	0.047	0.061	0.085	0.067	0.055	0.053	0.06
3	10	0.49	0.494	0.5	0.418	0.368	0.257	0.194	0.131
4	10	0.463	0.494	0.528	0.476	0.444	0.352	0.242	0.194

(continued)

TABLE 7 (continued)

Group	Number of animals assayed	Mean O.D. $_{414}$ readings of serum samples at serum dilutions of 1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600
Day 84									
1	10	0.03	0.04	0.03	0.03	0.03	0.03	0.03	0.03
3	10	0.26	0.29	0.27	0.25	0.19	0.15	0.10	0.07
4	10	0.26	0.28	0.28	0.27	0.22	0.17	0.12	0.09
Day 136									
1	pool	0.016	0.016	0.019	0.023	0.02	0.02	0.025	ND
3	pool	0.261	0.278	0.285	0.249	0.179	0.114	0.073	0.035
4	pool	0.254	0.274	0.271	0.261	0.224	0.161	0.118	0.095

[a]3.0 mg unencapsulated JE vaccine administered IP on Days 0, 14, and 42.

[b]3.0 mg unencapsulated JE vaccine, 3.0 mg microencapsulated JE vaccine prepared with 50:50 DL-PLG excipient, and 3.0 mg microencapsulated JE vaccine prepared with 65:35 DL-PLG excipient administered IP on Day 0.

[c]Untreated controls.

[d]ND = Not determined.

TABLE 8. ELISA RESULTS FROM THE JE VACCINE IMMUNIZATION STUDIES

Group	Treatment	Percent of standard serum, Day							
		14	21	35	44	63	77	84	136
1[a]	Control	ND[b]	1.7	0	10.6	10.3	15	15	6.9
3[c]	Unencapsulated JE	53.2	87.9	96.1	119.5	177.9	122.9	119	107.4
4[d]	JE microcapsules	39.9	67.7	102.7	144.6	157.7	129.8	124	104.6

[a]Untreated controls.

[b]ND = Not determined.

[c]3.0 mg unencapsulated JE vaccine administered IP on Days 0, 14, and 42.

[d]3.0 mg unencapsulated JE vaccine, 3.0 mg microencapsulated JE vaccine prepared with 50:50 DL-PLG excipient (microcapsule Batch E263-051), and 3.0 mg microencapsulated JE vaccine prepared with 65:35 DL-PLG excipient (microcapsule Batch E263-055) administered IP on Day 0.

TABLE 9. RESULTS OF CPE INHIBITION ASSAYS ON SERUM SAMPLES FROM THE JE VACCINE IMMUNIZATION STUDIES

Animal	Dilution of serum capable of reducing virus-induced CPE by 50% on Day 21	49	77
Group 1 = Untreated Controls			
A-1	<10	<10	<10
A-2	<10	13	<10
A-3	<10	<10	<10
A-4	<10	16	10
A-5	<10	<10	<20
B-1	<10	20	<10
B-2	<10	<10	<10
B-3	<10	10	13
B-4	<10	10	<10
B-5	<10	<10	<10
GMT[a]	<10	11	11
Average	<10	11	11
Maximum	<10	16	<20
Minimum	<10	<10	<10
Group 2 = 3.0 mg unencapsulated JE vaccine IP on Day 0			
A-1	<10	13	<10
A-2	50	127	80
A-3	25	63	32
A-4	127	160	160
A-5	20	40	10
B-1	80	254	101
B-2	40	64	40
B-3	80	80	50
B-4	80	50	101
B-5	40	101	127
GMT	44	73	50
Average	55	95	71
Maximum	127	254	160
Minimum	<10	13	<10

(continued)

TABLE 9 (continued)

Animal	Dilution of serum capable of reducing virus-induced CPE by 50% on Day 21	49	77
Group 3 = 3.0 mg unencapsulated JE vaccine IP on Days 0, 14, and 42			
A-1	4,064	>10,240	4,064
A-2	160	>10,240	254
A-3	1,280	>10,240	>8,127
A-4	320	1,280	403
A-5	320	2,032	1,280
B-1	160	806	1,280
B-2	ND[b]	3,325	1,613
B-3	ND	6,451	1,613
B-4	806	6,451	>10,240
B-5	403	2,560	640
GMT	507	3,880	1,576
Average	939	5,363	2,951
Maximum	4,064	>10,240	>10,240
Minimum	160	806	254
Group 4 = Prototype microcapsule system IP on Day 0			
A-1	101	5,120	5,120
A-2	101	320	1,016
A-3	13	160	254
A-4	32	1,016	1,613
A-5	320	1,016	3,225
B-1	64	640	1,280
B-2	64	403	1,016
B-3	127	2,560	10,240
B-4	80	806	403
B-5	127	254	508

(continued)

TABLE 9 (continued)

Animal	Dilution of serum capable of reducing virus-induced CPE by 50% on Day		
	21	49	77
GMT	77	718	1,341
Average	103	1,230	2,468
Maximum	320	5,120	10,240
Miminum	13	160	254

[a]GMT = Geometric mean titers.

[b]Not determined. (No Sample)

TABLE 10. RESULTS OF PLAQUE-REDUCTION ASSAYS ON POOLED SERUM SAMPLES FROM JE VACCINE IMMUNIZATION STUDIES

Group	Treatment	Day	Serum dilution to reach 50% endpoint	Serum dilution to reach 80% endpoint
1[a]	Controls	0	<10	<10
1	Controls	14	<10	<10
1	Controls	21	<10	<10
1	Controls	42	<10	<10
1	Controls	49	<10	<10
1	Controls	84	<10	<10
2[b]	Unencapsulated JE	0	<10	<10
2	Unencapsulated JE	14	160	20
2	Unencapsulated JE	21	ND[c]	ND
2	Unencapsulated JE	42	320	80
2	Unencapsulated JE	49	320	40
2	Unencapsulated JE	84	640	160
3[d]	Unencapsulated JE	0	<10	<10
3	Unencapsulated JE	14	160	40
3	Unencapsulated JE	21	2,560	640
3	Unencapsulated JE	42	1,280	640
3	Unencapsulated JE	49	5,120	2,560
3	Unencapsulated JE	84	2,560	1,280
4[e]	Microencapsulated JE	0	<10	<10
4	Microencapsulated JE	14	160	20
4	Microencapsulated JE	21	320	80
4	Microencapsulated JE	42	5,120	640
4	Microencapsulated JE	49	5,120	640
4	Microencapsulated JE	84	10,000	2,560

[a]Untreated controls.

[b]Animals received 3.0 mg of unencapsulated JE vaccine IP on Day 0.

[c]ND = Not determined (insufficient sample quantity).

[d]Animals received 3.0 mg of unencapsulated JE vaccine IP on Day 0, 14, and 42.

[e]Animals received 3.0 mg of unencapsulated and 6.0 mg of microencapsulated JE vaccine IP on Day 0.

TABLE 11. EFFECT OF JE VIRUS (BEIJING STRAIN) CHALLENGE ON CD-1 MICE

Challenge, pfu IP	Mortality, dead/total
Uninfected	0/10
500	2/10
250	0/10
100	1/10
50	0/10
25	0/10
10	0/10
5	0/10
1	0/10
0.5	0/10
0.1	0/10
0.05	0/10

TABLE 12. PROPERTIES OF MURAMYL TRIPEPTIDE MICROCAPSULES PREPARED WITH DL-PLG EXCIPIENTS

Microcapsule Batch	Polymer Batch	Yield, % of theoretical	Core loading, wt % MTP		Encapsulation efficiency, % of theoretical	MTP released in vitro, %, at			
			Theoretical	Actual		1 h	6 h	24 h	48 h
D820-013	C779-090[a]	103.0	2.01	1.0	49.8	24.5	82.2	105.7	110.9
D820-039	D466-027[b]	ND[c]	5.04	4.6	91.3	46.7	69.7	80.7	84.9
D820-054	C779-154[d]	67.0	2.06	1.3	63.1	78.1	103.5	114.0	ND
D820-057	C779-154	84.2	3.85	2.7	70.1	38.7	61.5	69.8	73.7
D820-062	C779-154	58.4	2.07	0.6	29.0	2.7	7.3	12.9	19.3
D820-086	C779-154	64.1	2.18	1.0	45.9	0.7	2.6	4.7	7.8
D820-089	C779-154	54.4	2.16	0.9	41.7	1.3	3.2	5.7	8.8
D820-105	C779-154	68.3	2.09	0.2	9.6	1.6	17.7	34.6	46.4
E529-044	D726-070[e]	69.0	2.14	0.4	18.7	0.0	0.3	5.3	11.9
E529-127	C779-154	66.3	2.21	1.8	81.4	1.2	3.9	4.5	5.2
E529-129	C779-154	66.3	2.13	2.3	108.0	0.6	2.7	3.2	3.7
E529-133	C779-154	69.4	2.02	1.6	79.2	1.9	5.2	5.9	6.7
E529-135	C779-154	77.1	2.19	1.3	59.4	2.6	6.8	7.7	8.4
E529-137	C779-154	74.2	2.14	1.4	65.4	2.3	6.7	7.6	8.4
E529-139	C779-154	76.1	2.19	1.2	54.8	2.7	6.8	7.9	9.1
E529-141	C779-154	--	--	1.3	--	0.8	6.8	12.9	12.9

[a]54:46 poly(DL-lactide-co-glycolide), IV = 0.83 dL/g, HFIP, 30 °C, 0.5 g/dL.

[b]50:50 poly(DL-lactide-co-glycolide), IV = 1.20 dL/g, HFIP, 30 °C, 0.5 g/dL.

[c]ND = Not determined.

[d]50:50 poly(DL-lactide-co-glycolide), IV = 0.63 dL/g, HFIP, 30 °C, 0.5 g/dL.

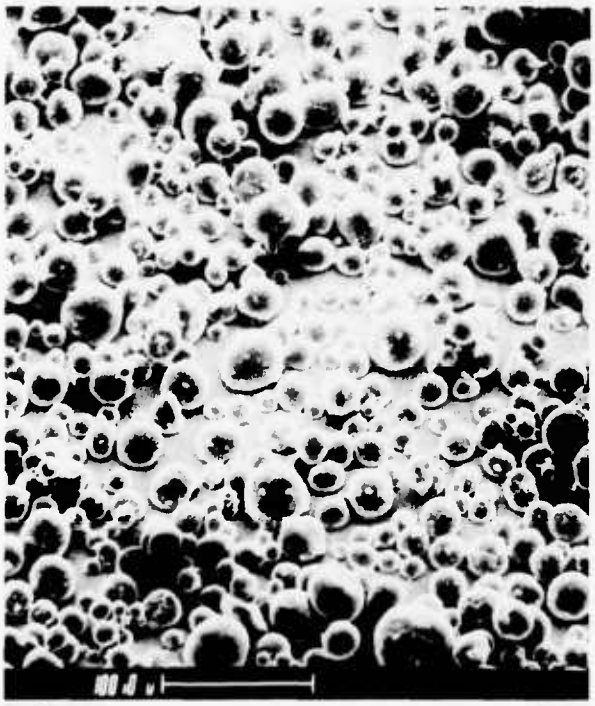
[e]65:35 poly(DL-lactide-co-glycolide), IV = 0.65 dL/g, HFIP, 30 °C, 0.5 g/dL.

APPENDIX B

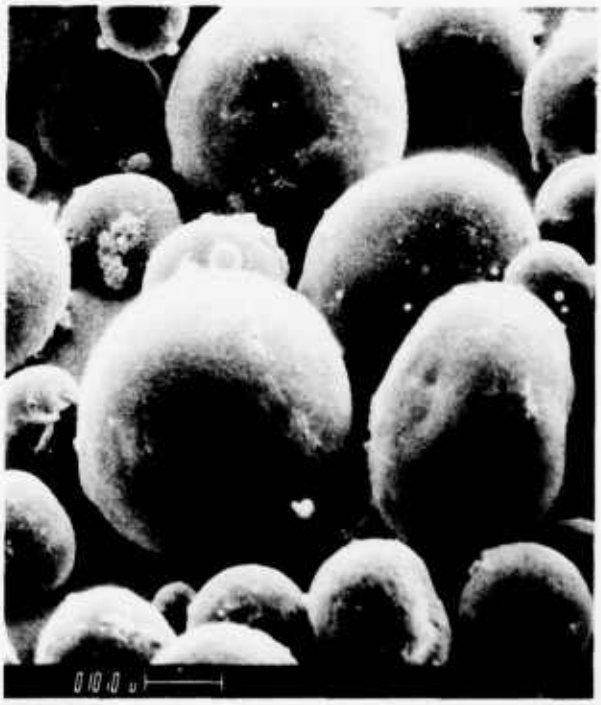
FIGURES

E100-019 1000X

E100-019 6000X



E100-020 200X



E100-020 1000X

6948-19

Figure 1. Photomicrographs taken by scanning electron microscopy of microcapsules made with the physiological salt of poly(I·C) and prepared with a DL-PLG excipient: Batches E100-019 and E100-020.

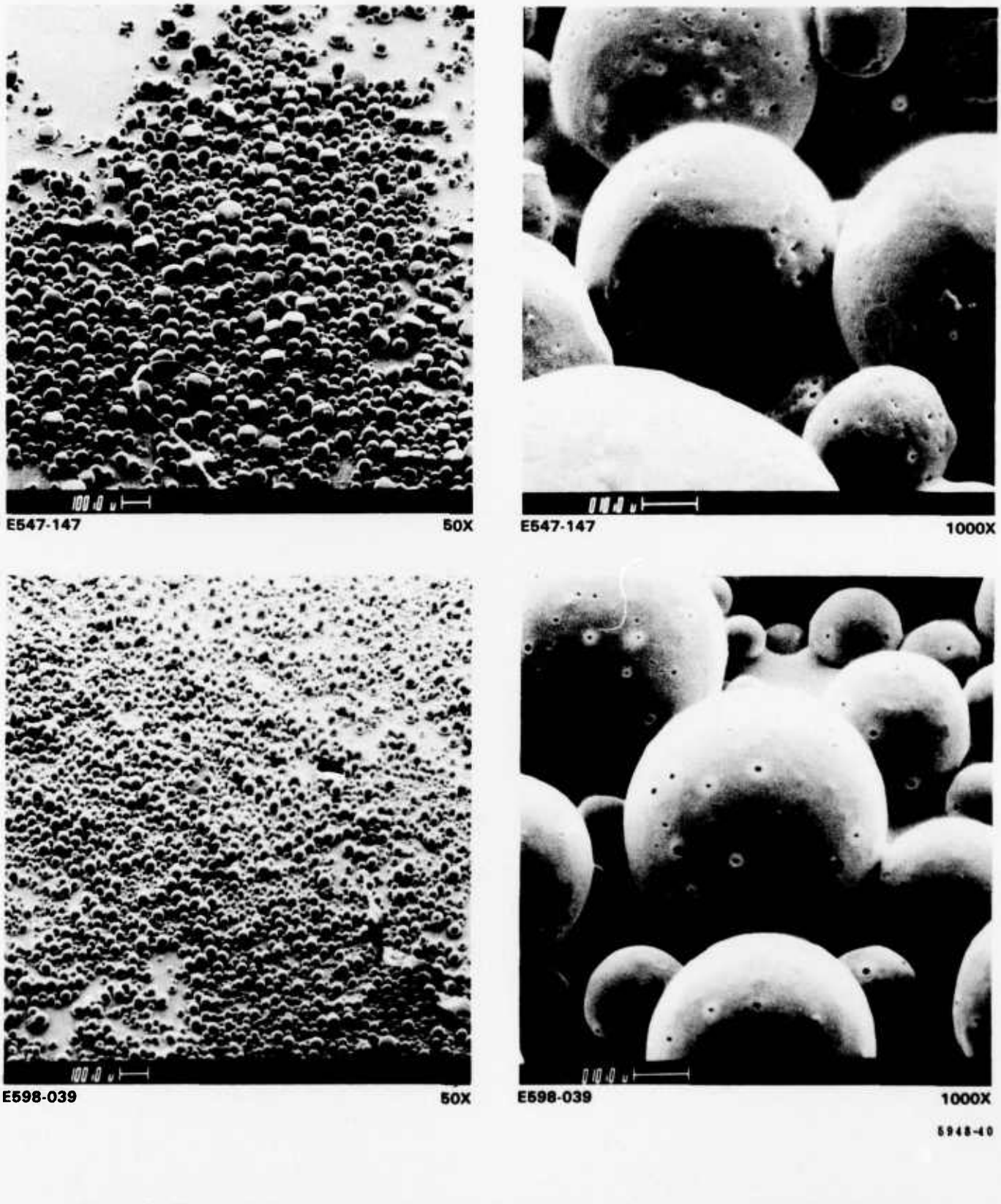


Figure 2. Photomicrographs taken by scanning electron microscopy of microcapsules made with the sodium salt of poly (I·C) and prepared with a DL-PLG excipient: Batches E547-147 and E598-039.

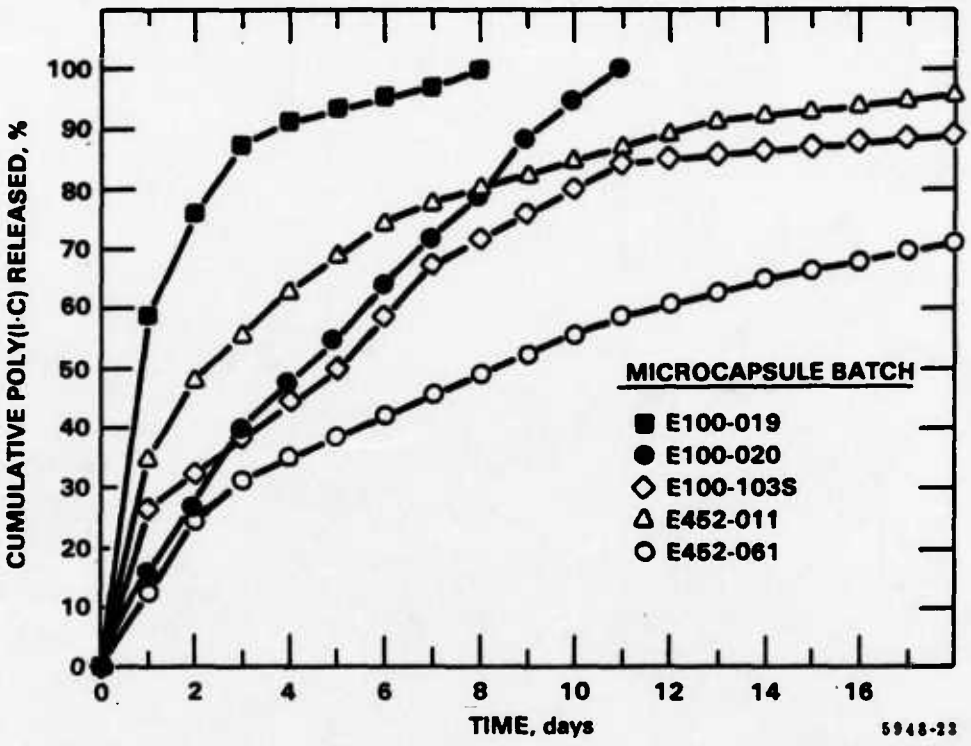


Figure 3. Cumulative in vitro release profiles of poly(I·C) from microcapsules prepared for in vivo studies (receiving fluid is 0.15 M NaCl; 35 °C).

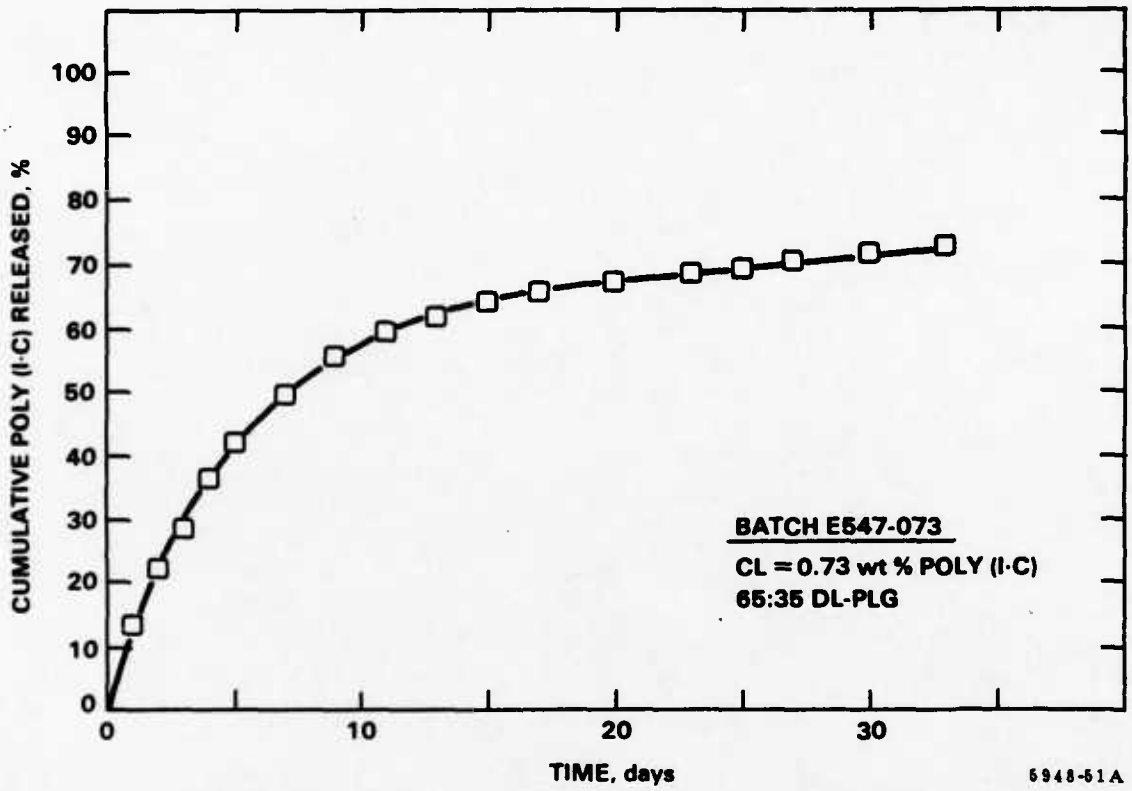


Figure 4. Cumulative in vitro release profile of poly(I·C) from microcapsules prepared in vivo studies: Batch E547-073 (receiving fluid is 0.15 M NaCl; 37°).

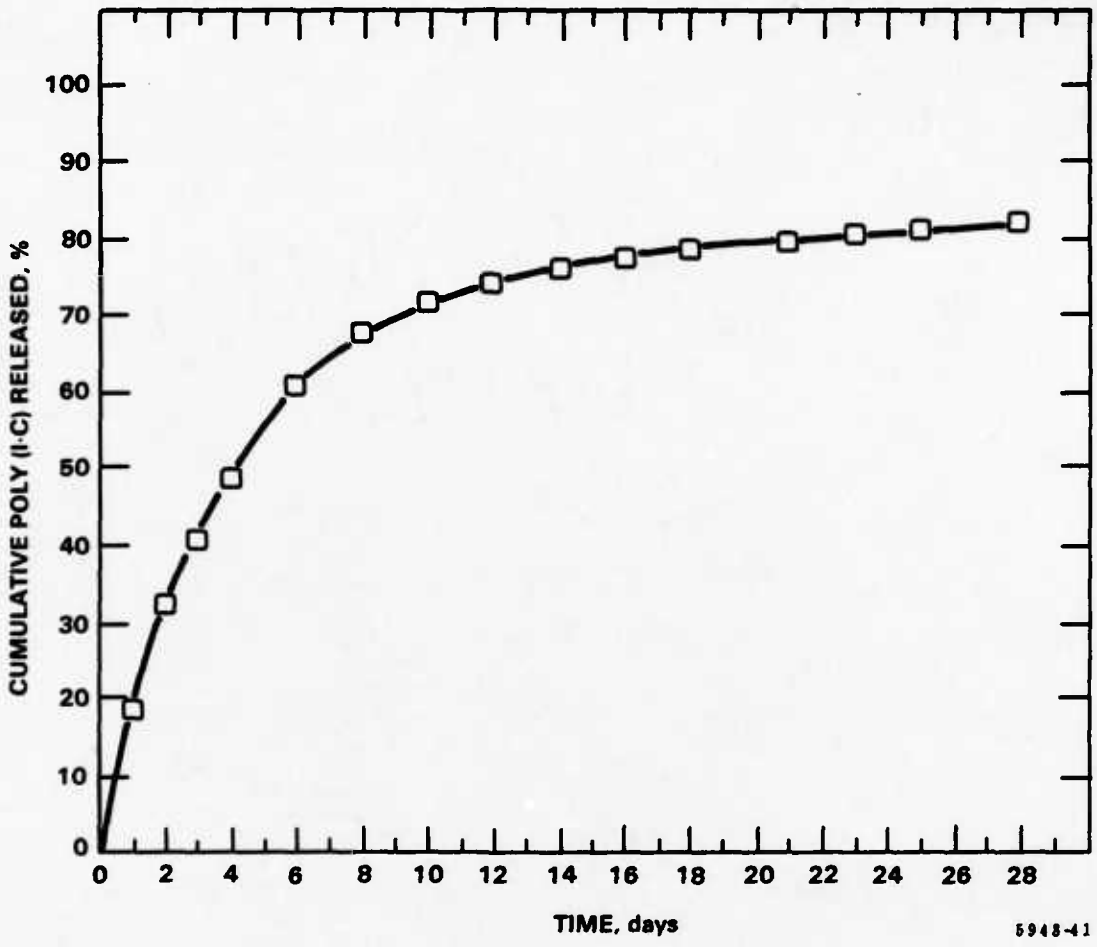


Figure 5. Cumulative in vitro release of poly(I·C) from 0.96%-loaded microcapsules: Batch E598-039. These microcapsules were prepared using the sodium salt of poly(I·C) (receiving fluid is 0.15 M NaCl; 37 °C).

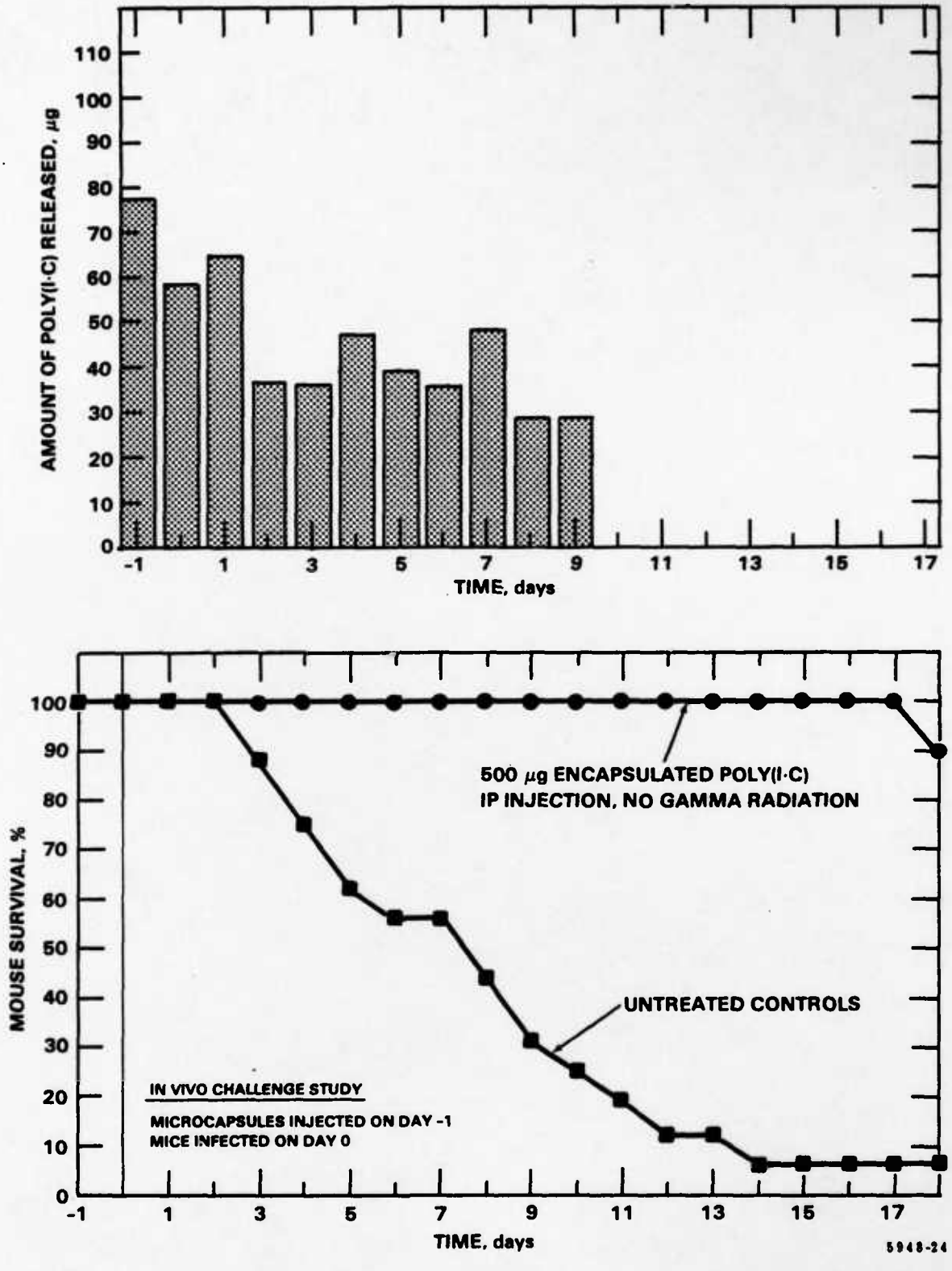


Figure 6. Daily in vitro release kinetics (above) and survival rate (below) for mice infected with Rift Valley Fever virus and treated IP with 500 µg of encapsulated poly(I·C): Batch E100-020.

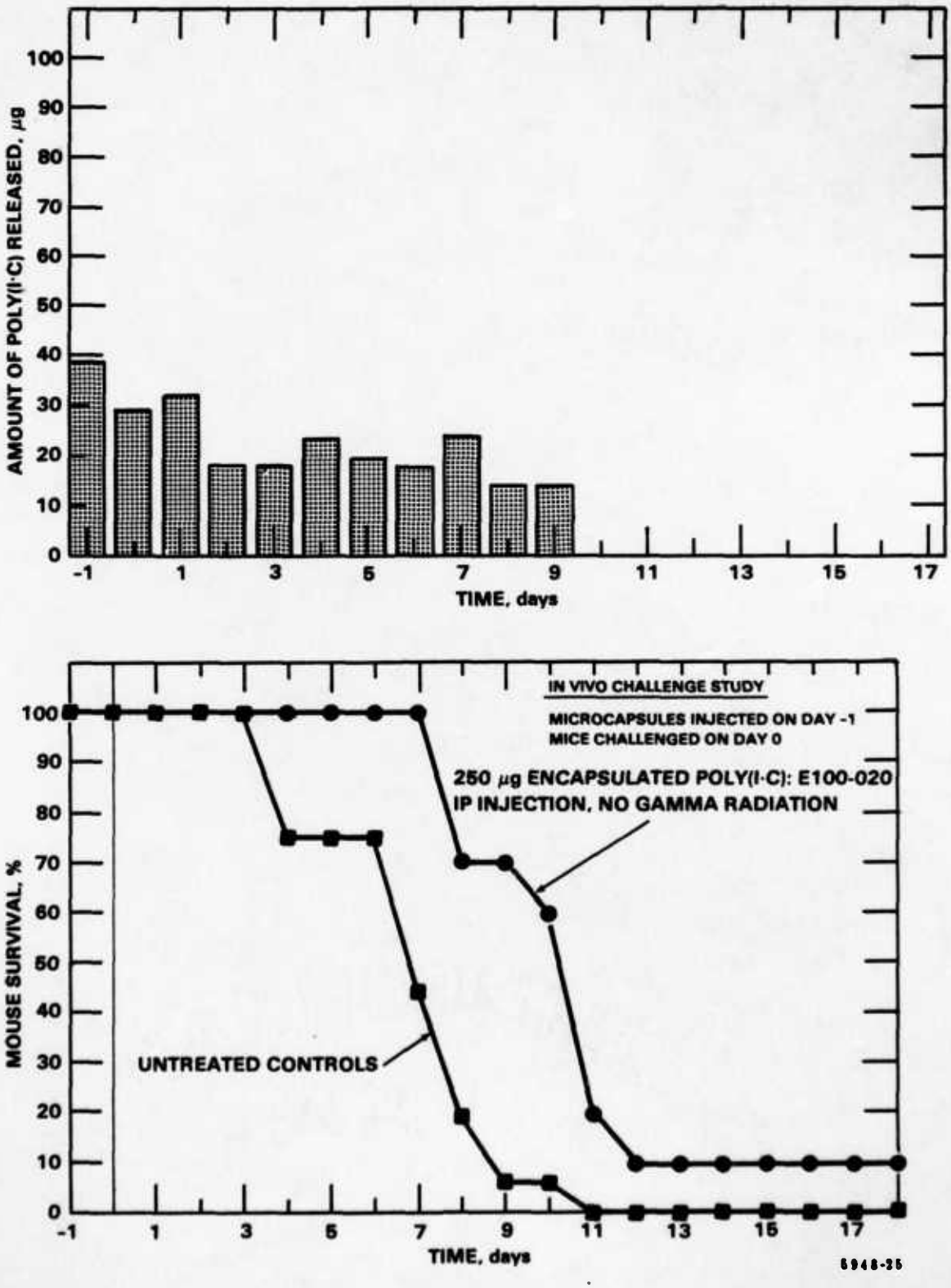


Figure 7. Daily in vitro release kinetics (above) and survival rate (below) for mice infected with Rift Valley Fever virus and treated IP with 250 µg of encapsulated poly(I·C): Batch E100-020.

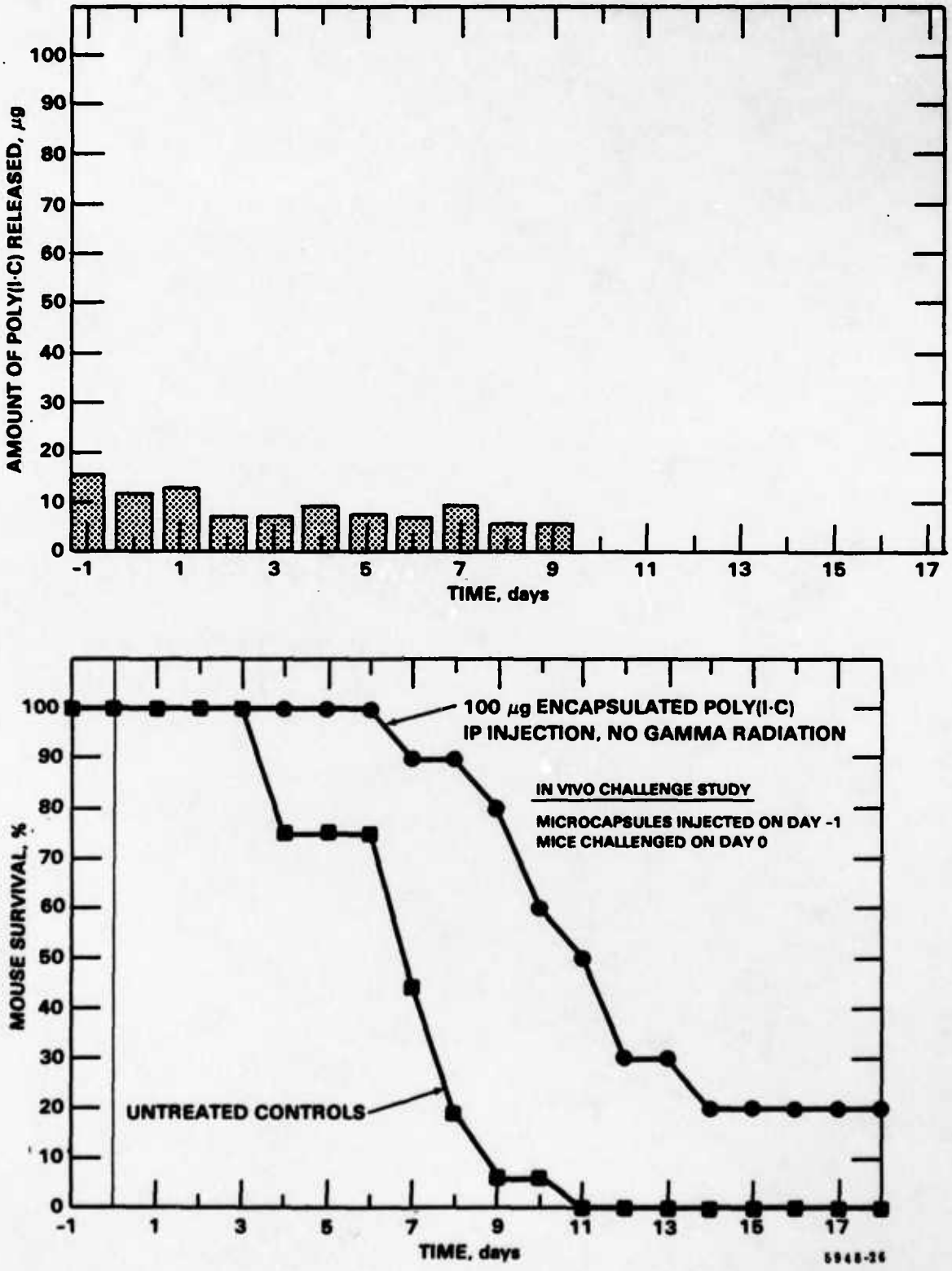


Figure 8. Daily in vitro release kinetics (above) and survival rate (below) for mice infected with Rift Valley Fever virus and treated IP with 100 µg of encapsulated poly(I·C): Batch E100-020.

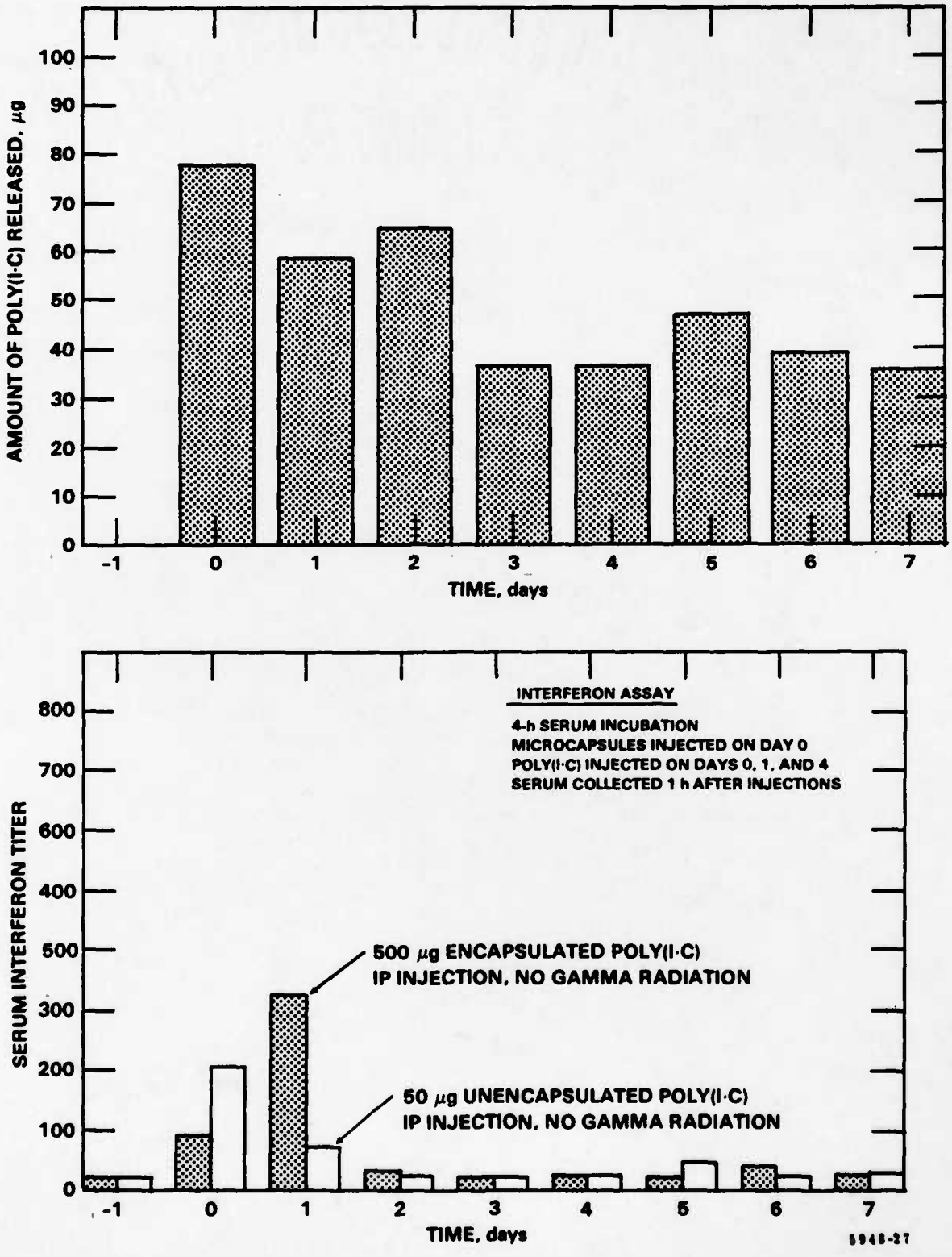


Figure 9. Daily in vitro release kinetics (above) and interferon levels (below) for mice treated IP with 500 µg of encapsulated poly(I·C): Batch E100-020.

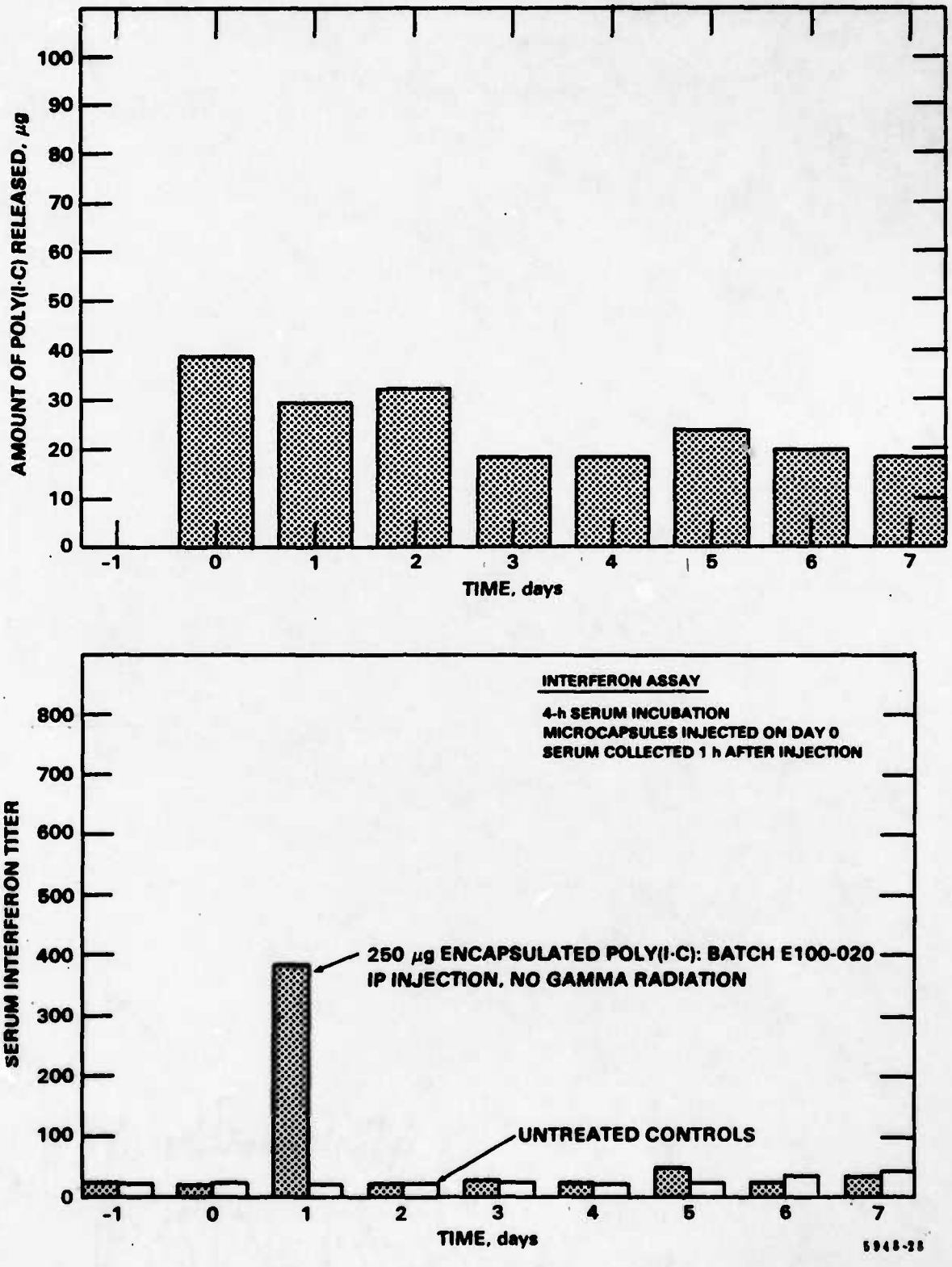


Figure 10. Daily in vitro release kinetics (above) and interferon levels (below) for mice treated IP with 250 μg of encapsulated poly(I·C): Batch E100-020.

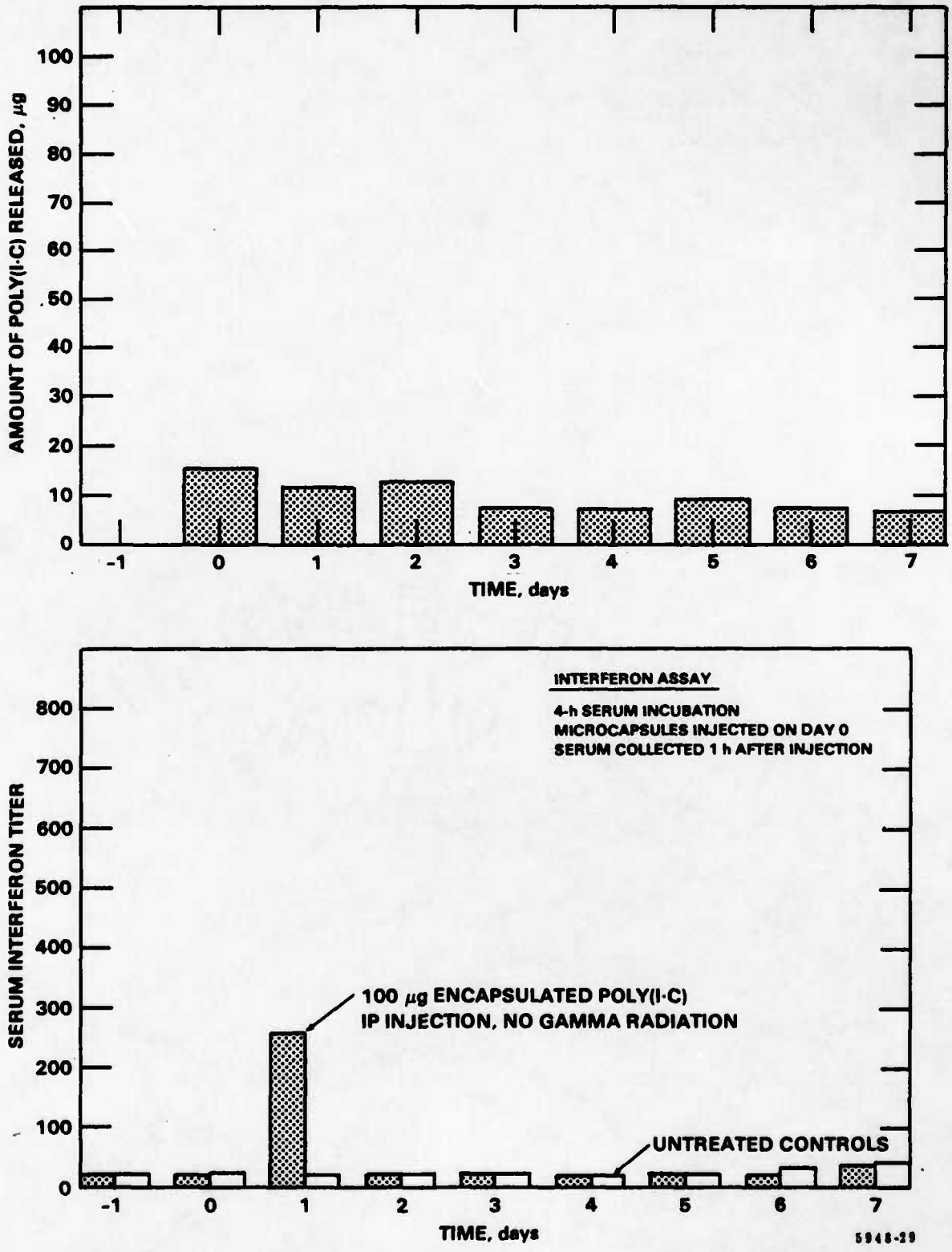


Figure 11. Daily in vitro release kinetics (above) and interferon levels (below) for mice treated IP with 100 μg of encapsulated poly(I·C): Batch E100-020.

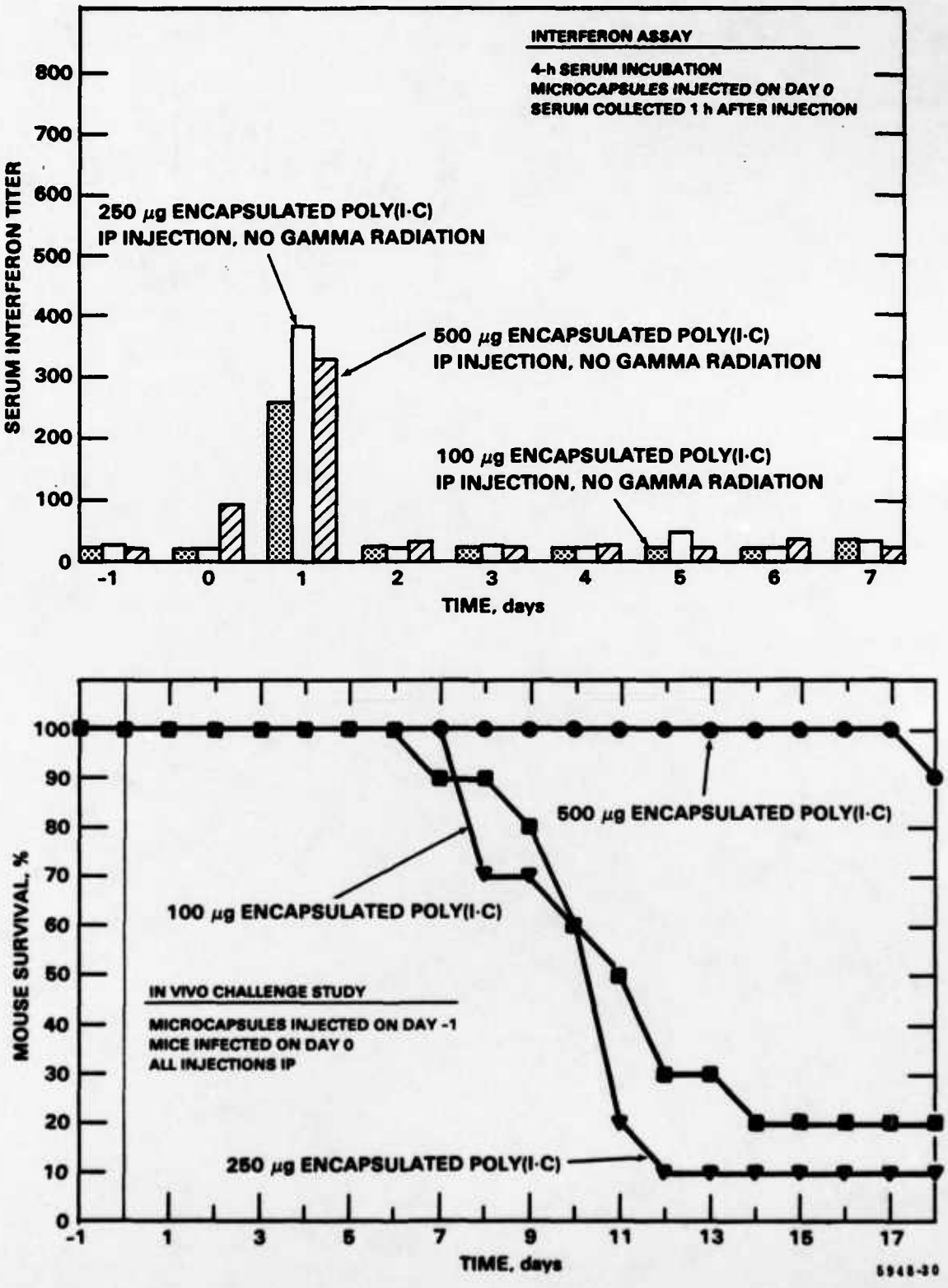


Figure 12. Daily interferon levels (above) and survival rate (below) for mice treated IP with various doses of encapsulated poly(I·C): Batch E100-020.

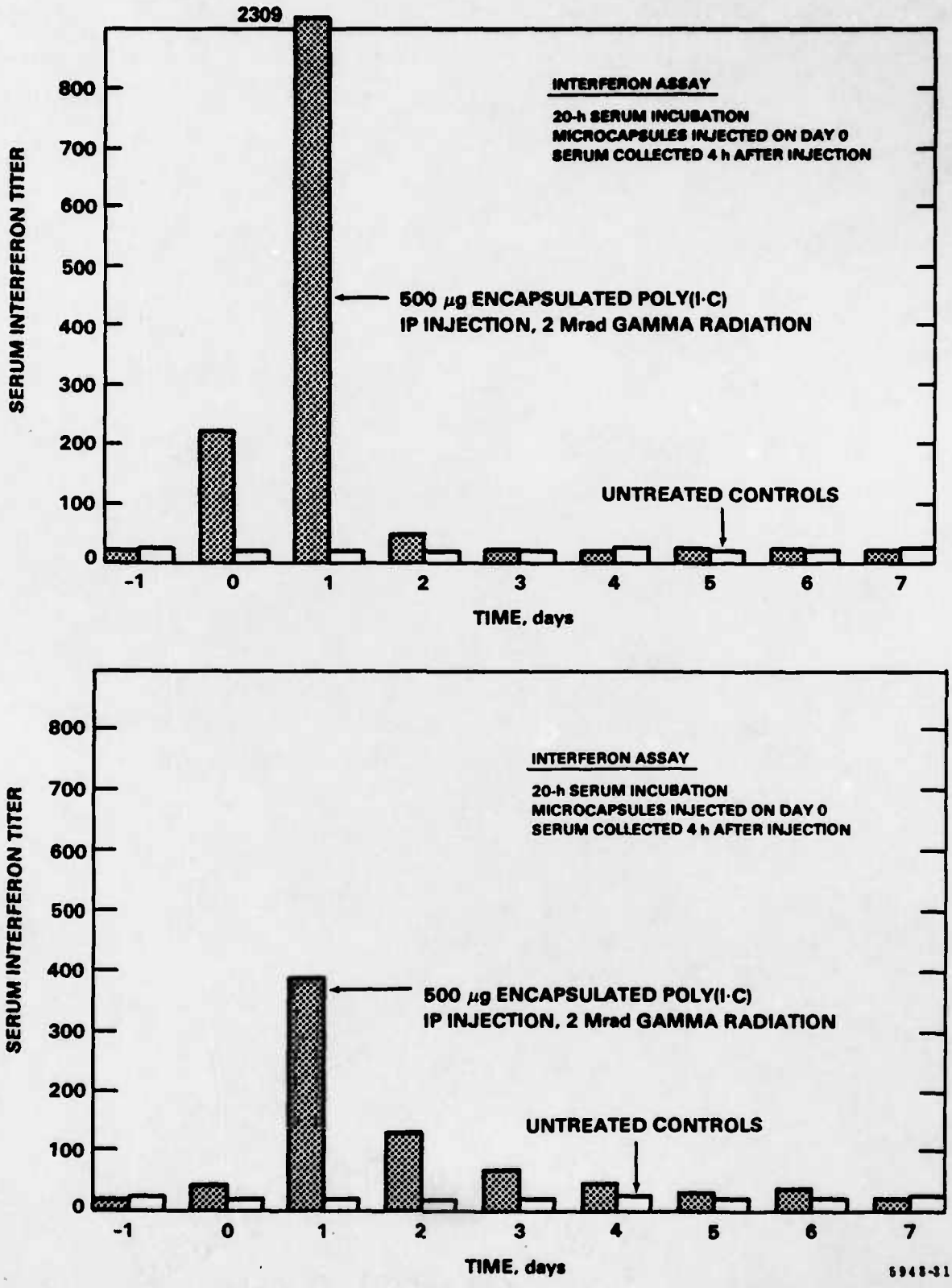


Figure 13. Daily interferon levels for mice treated IP (above) and SC (below) with 500 μg of sterilized encapsulated poly(I·C): Batch E100-103S.

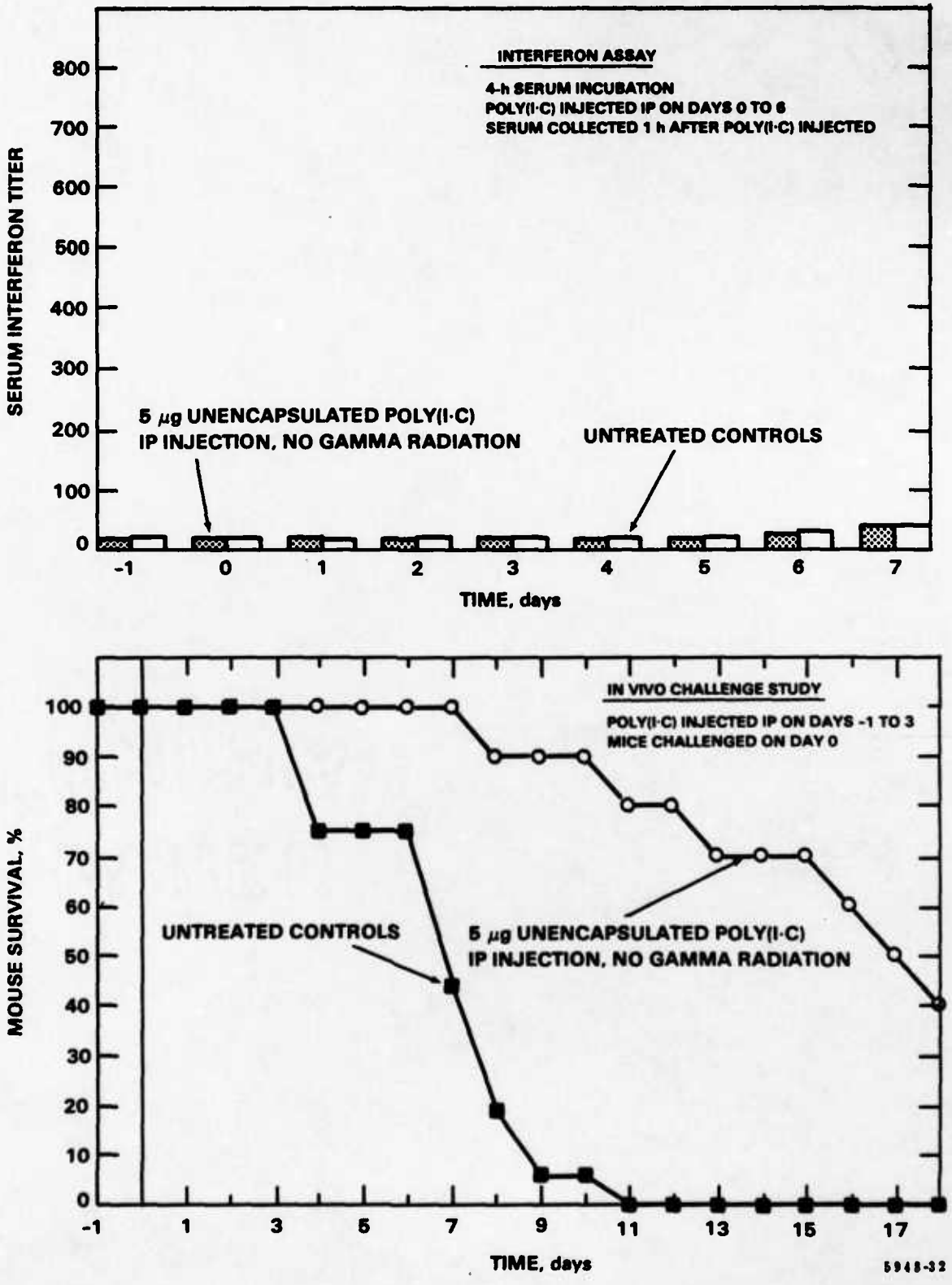


Figure 14. Daily interferon levels (above) and survival rate (below) for mice infected with Rift Valley Fever virus and treated IP with multiple injections of 5 μg of unencapsulated poly(I·C).

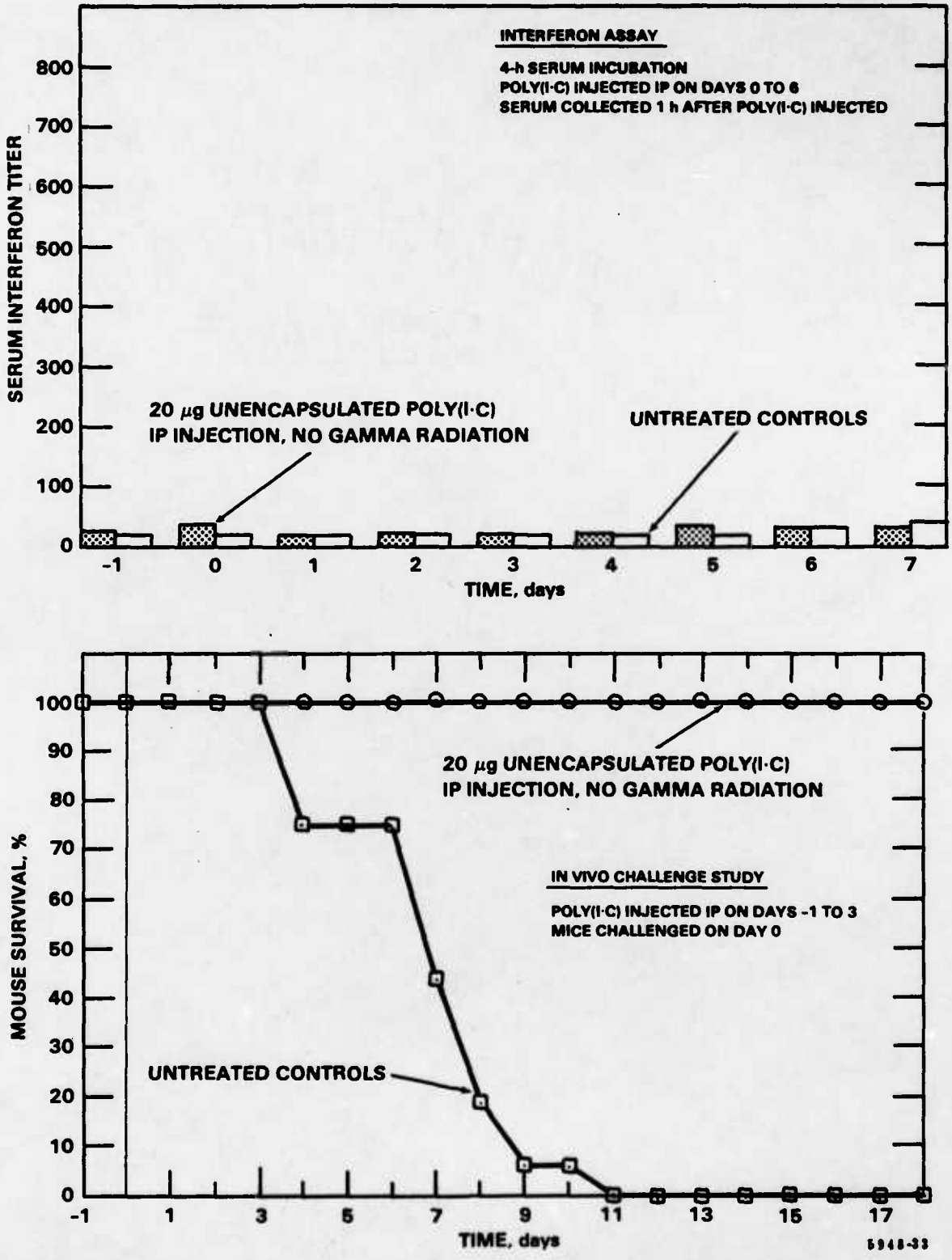


Figure 15. Daily interferon levels (above) and survival rate (below) for mice infected with Rift Valley Fever virus and treated IP with multiple injections of 20 μg of unencapsulated poly(I·C).

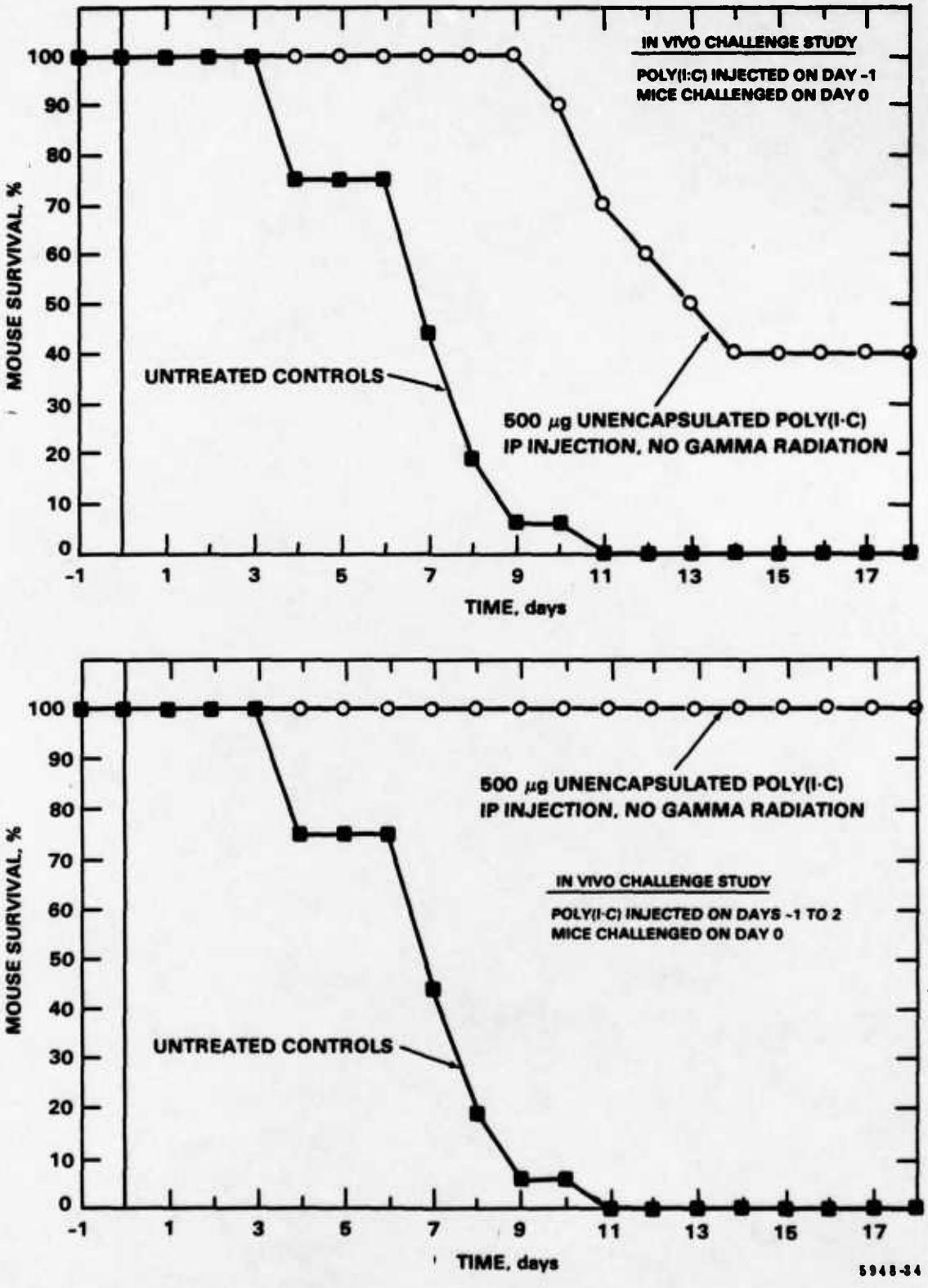


Figure 16. Survival rates for mice infected with Rift Valley Fever virus and treated IP with a single injection (above) and multiple injections (below) of 500 μg of unencapsulated poly(I·C).

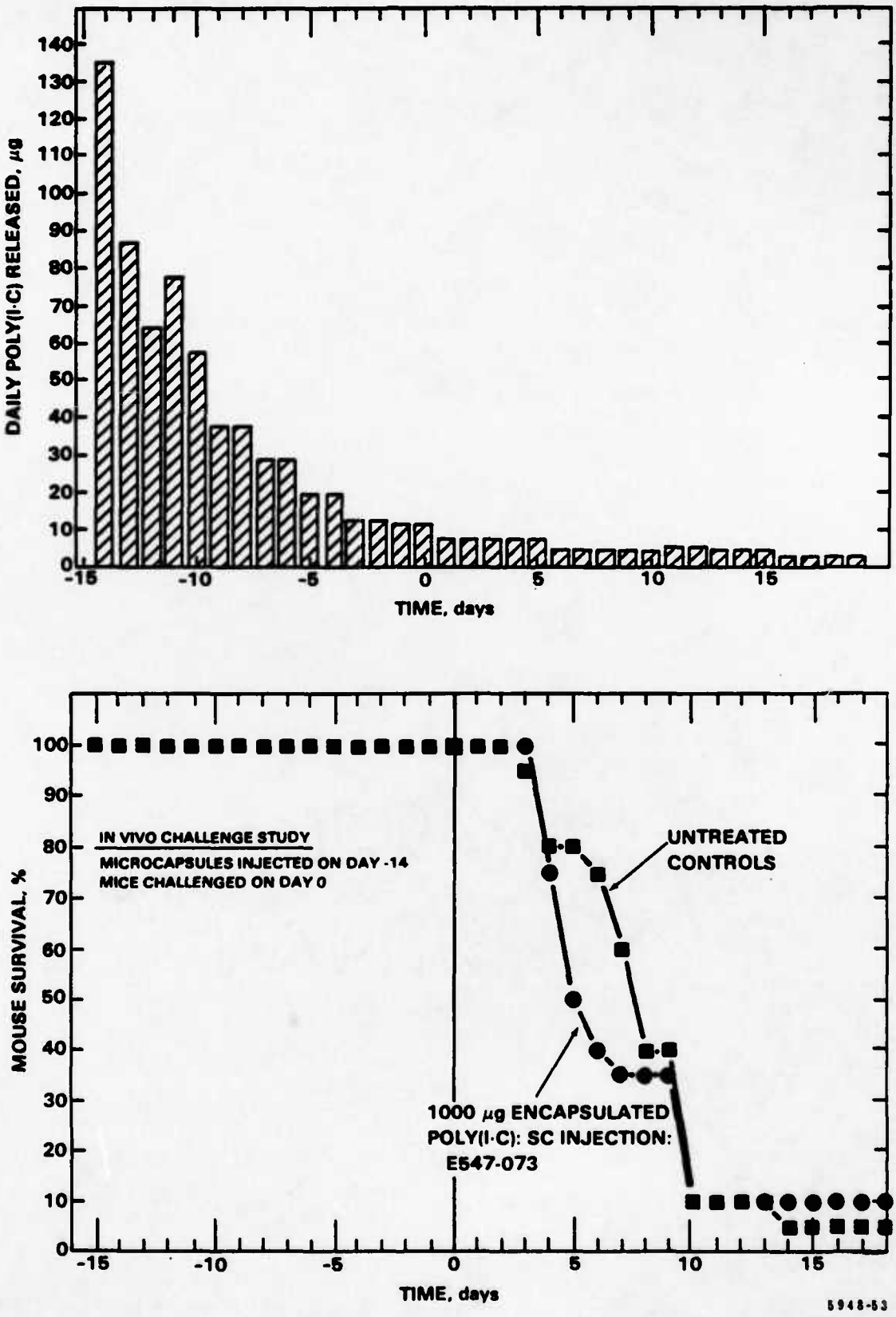


Figure 17. Daily in vitro release kinetics (above: 0.15 M NaCl; 37 °C) and survival rate (below) for mice infected with Rift Valley Fever virus and treated SC with 1000 µg of encapsulated poly (I·C): Batch E547-073.

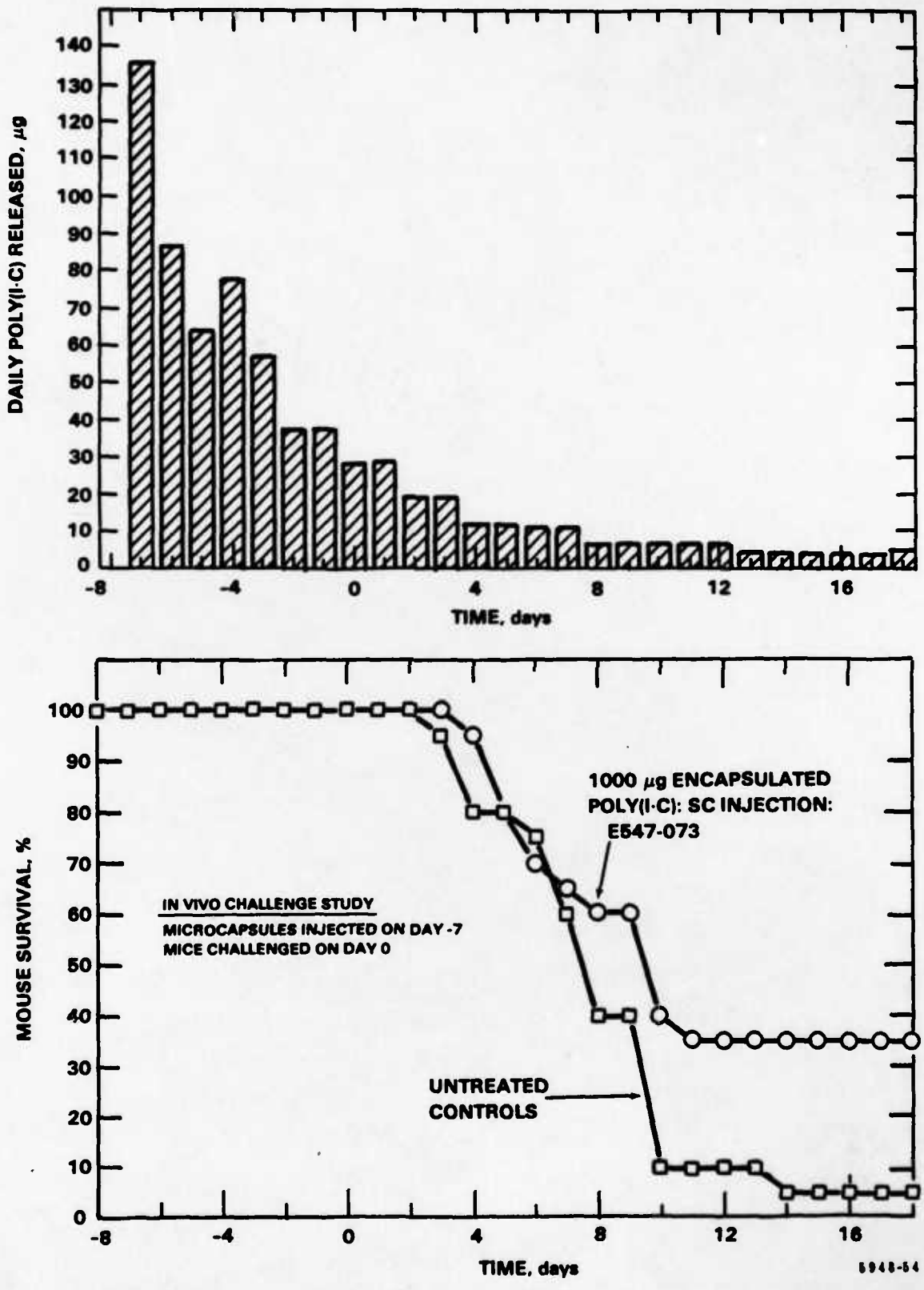


Figure 18. Daily in vitro release kinetics (above: 0.15 M NaCl; 37 °C) and survival rate (below) for mice infected with Rift Valley Fever virus and treated SC with 1000 µg of encapsulated poly (I·C): Batch E547-073.

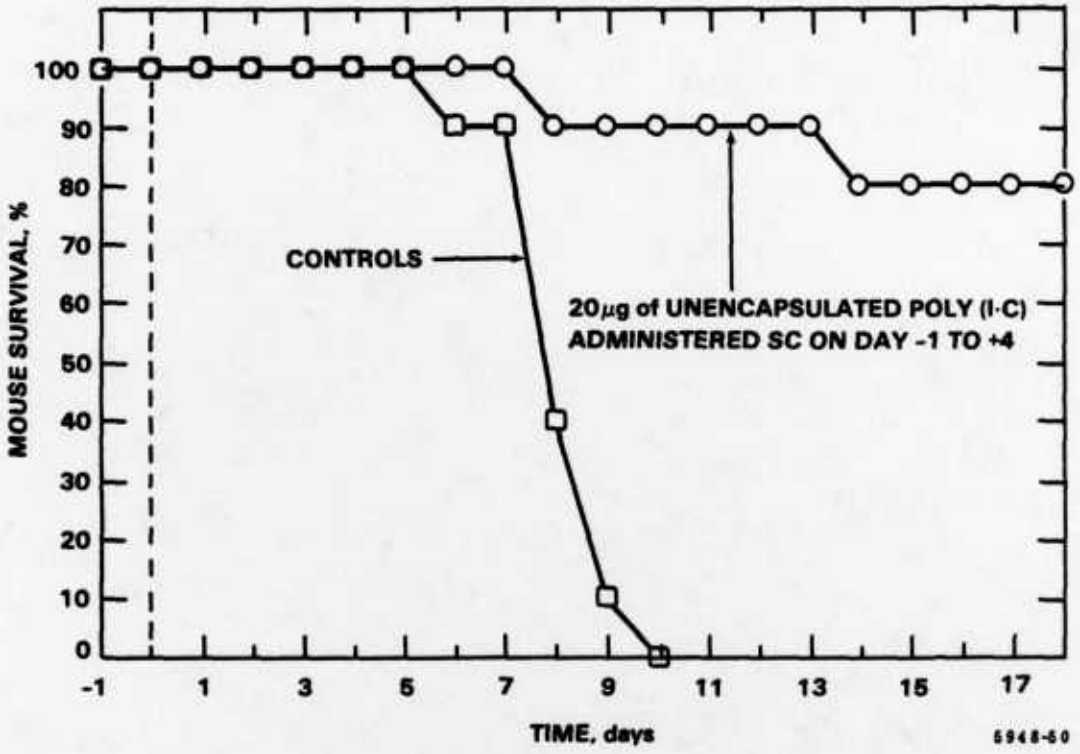


Figure 19. Survival rate for mice infected with VEE virus and injected with 20 μg of unencapsulated poly (I·C) on Day -1 to +4. Control animals were injected with phosphate-buffered saline solution on Day -1 to +4.

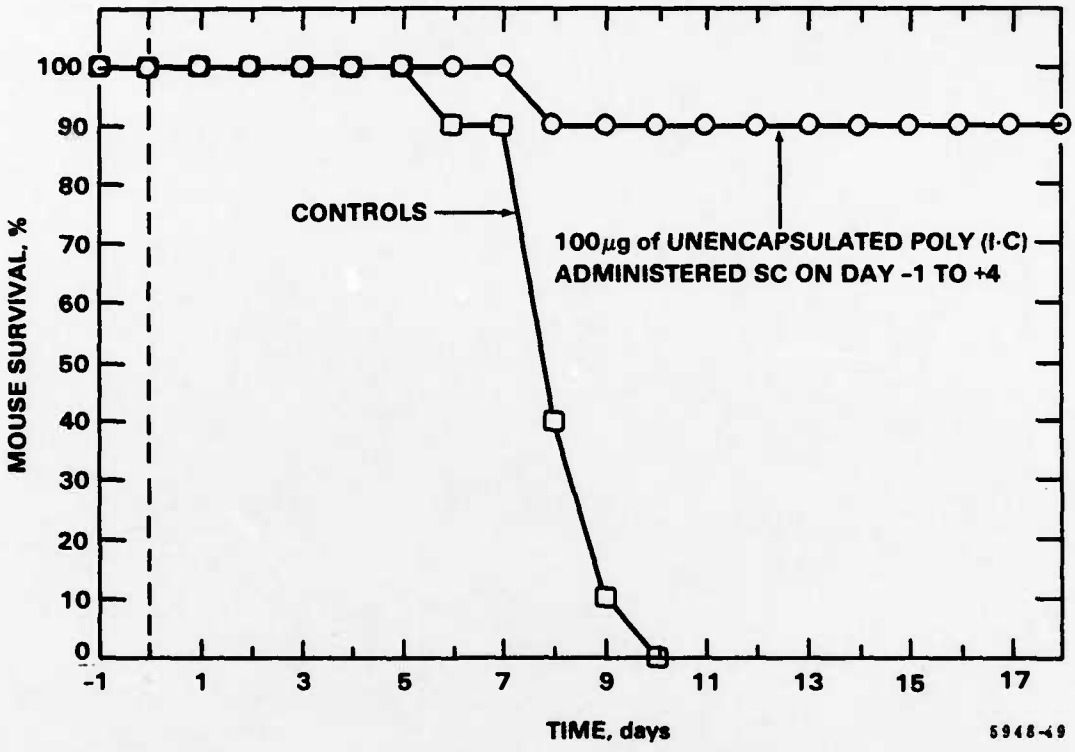


Figure 20. Survival rate for mice infected with VEE virus and injected with 100 μg of unencapsulated poly (I·C) on Day –1 to +4. Control animals were injected with phosphate-buffered saline solution on Day –1 to +4.

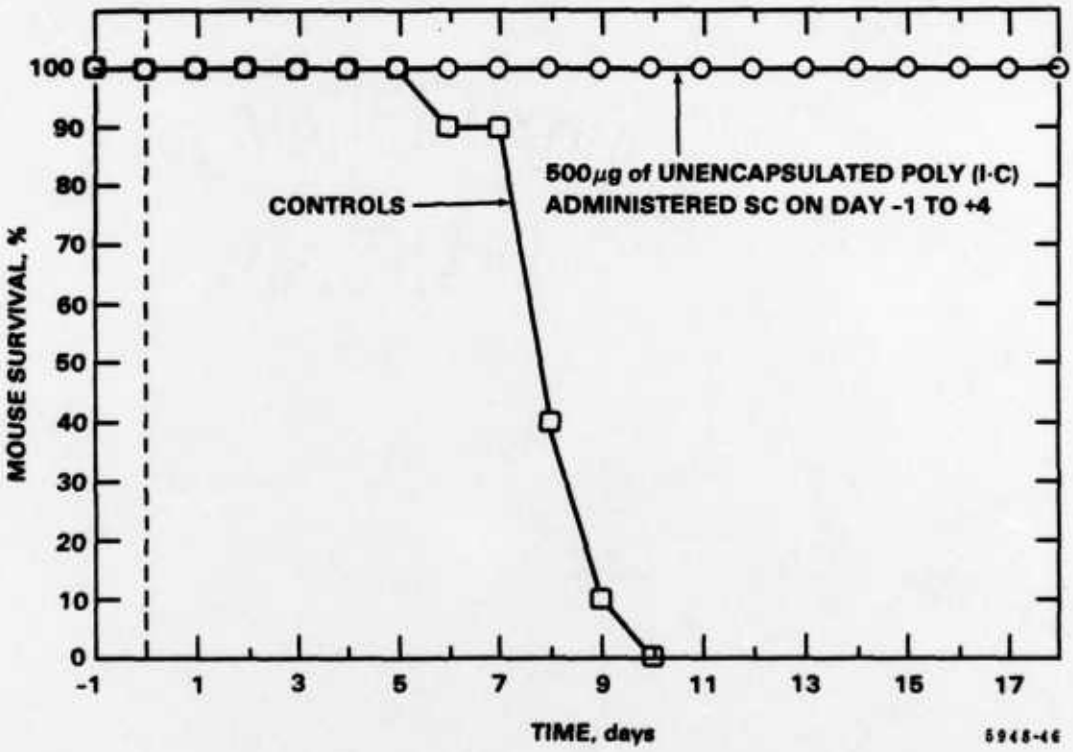


Figure 21. Survival rate for mice infected with VEE virus and injected with 500 μg of unencapsulated poly (I·C) on Day -1 to +4. Control animals were injected with phosphate-buffered saline solution on Day -1 to +4.

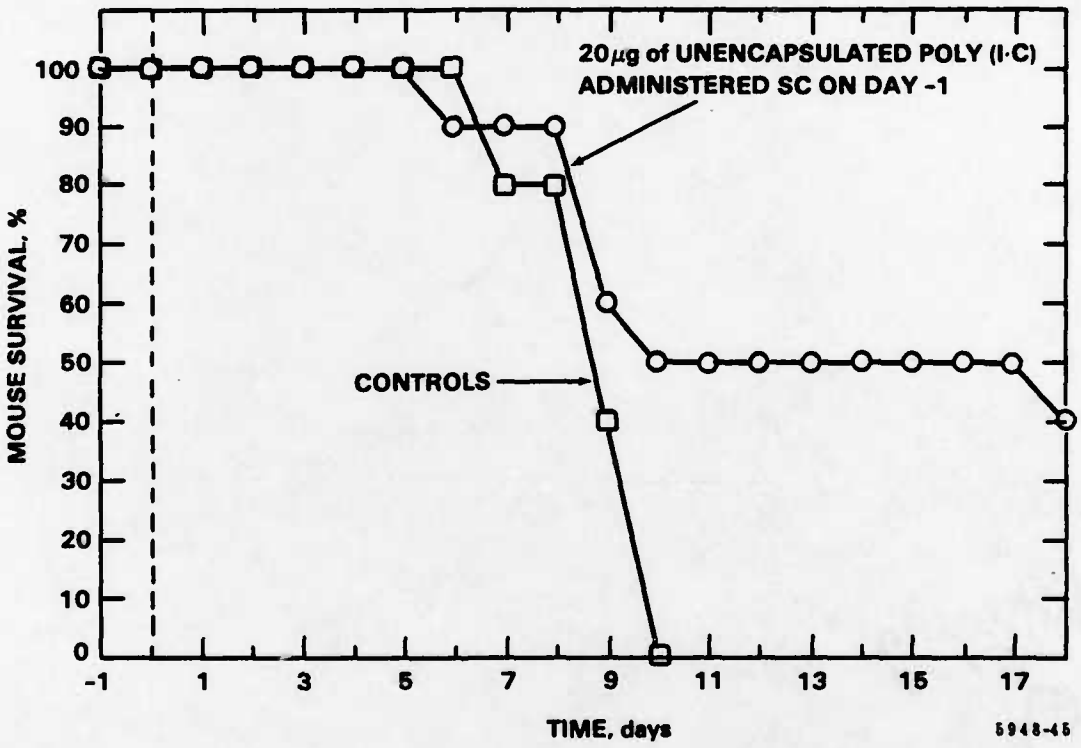


Figure 22. Survival rate for mice infected with VEE virus and injected with 20 μg of unencapsulated poly (I·C) on Day -1. Control animals were injected with phosphate-buffered saline solution on Day -1.

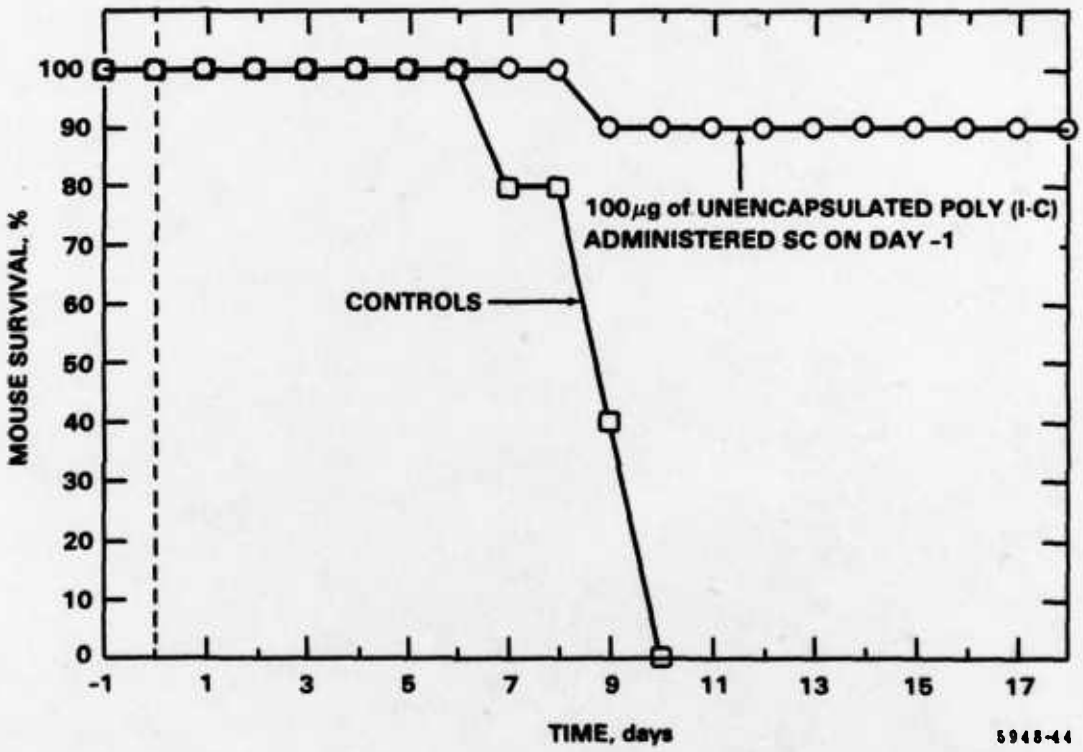


Figure 23. Survival rate for mice infected with VEE virus and injected with 100 μg of unencapsulated poly (I·C) on Day –1. Control animals were injected with phosphate-buffered saline solution on Day –1.

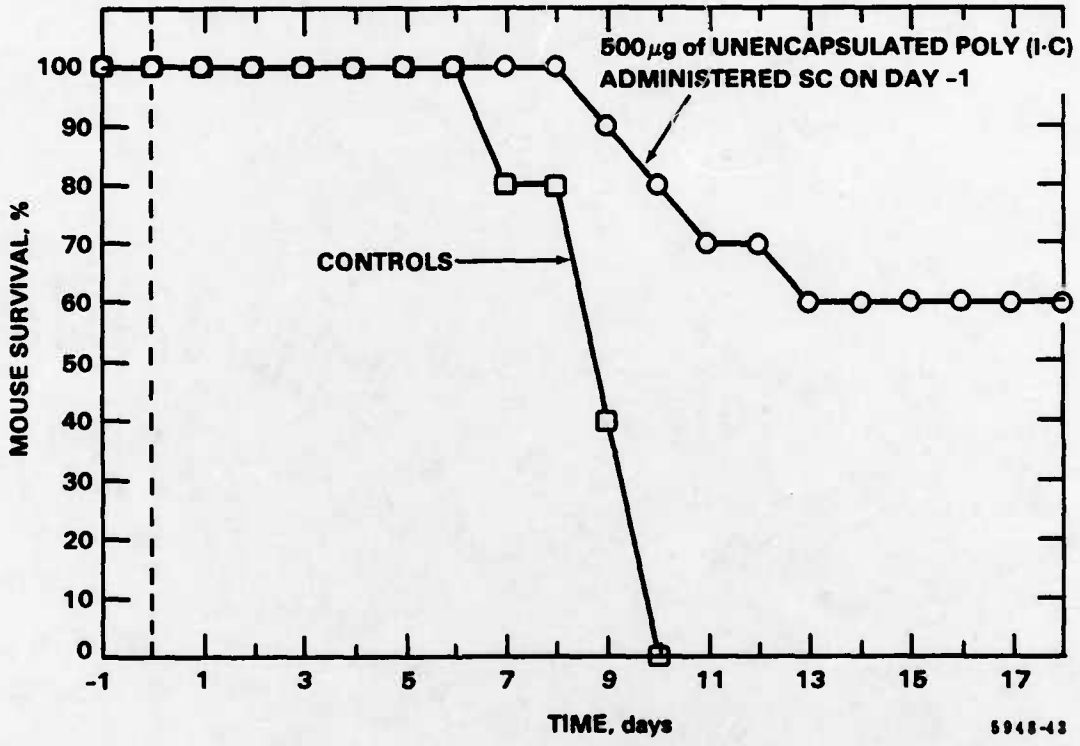


Figure 24. Survival rate for mice infected with VEE virus and injected with 500 μg of unencapsulated poly (I·C) on Day -1. Control animals were injected with phosphate-buffered saline solution on Day -1.

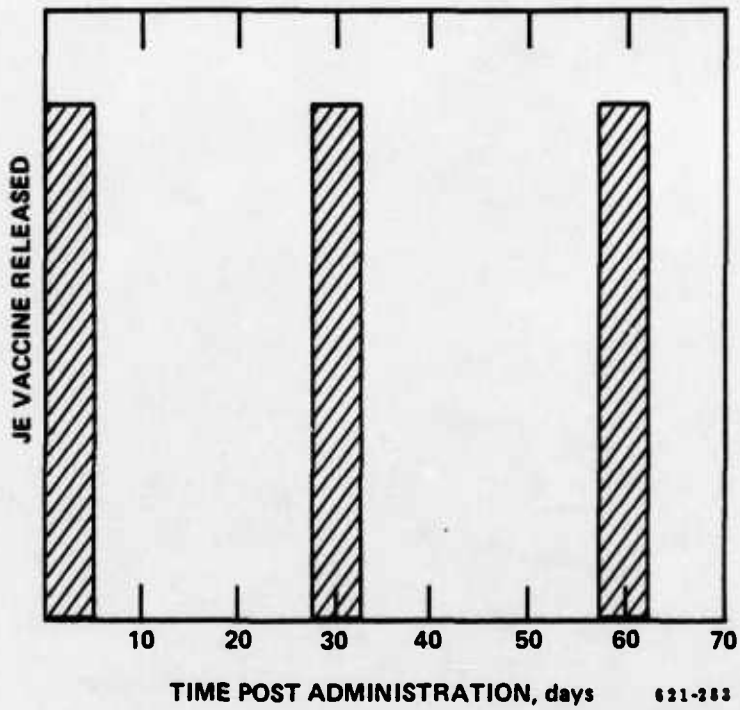
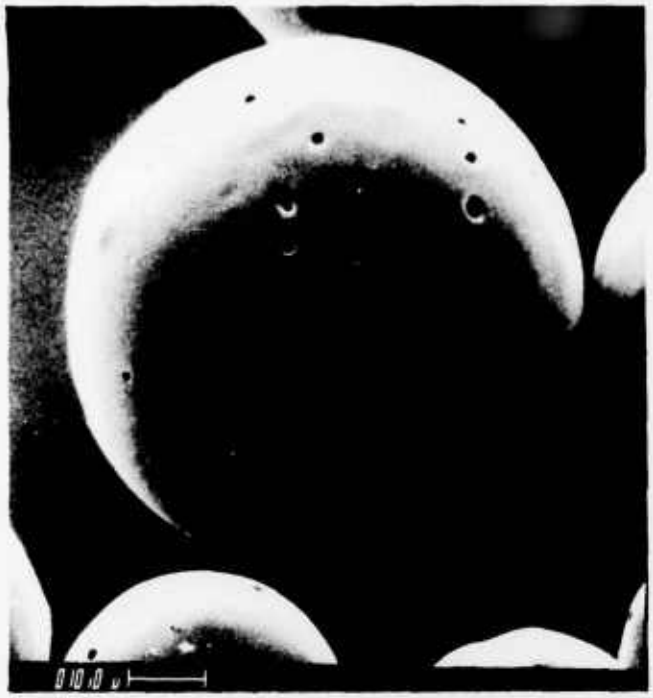


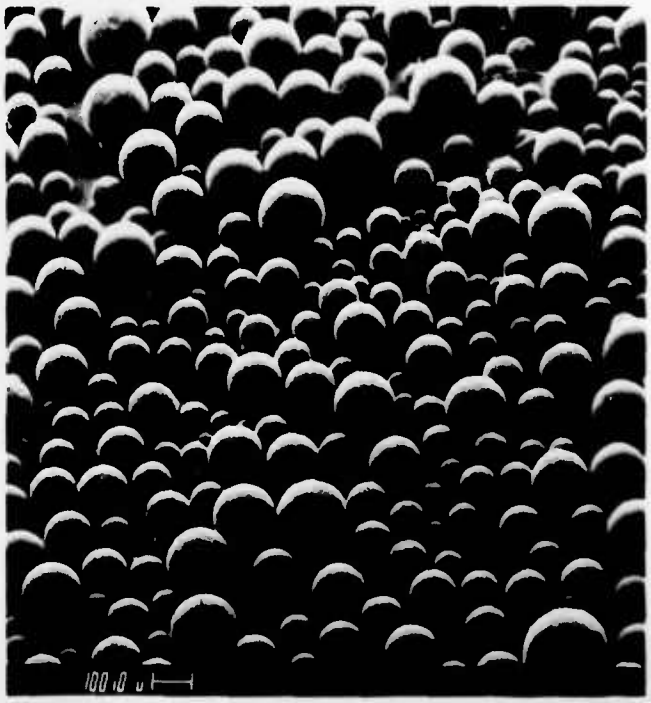
Figure 25. Diagram demonstrating the release profile that could be obtained for a programmed-delivery microcapsule formulation consisting of JE vaccine encapsulated in various DL-PLG excipients. The formulation would release unencapsulated JE vaccine initially after administration to elicit a primary response. No additional vaccine would then be released until sufficient time had elapsed to obtain efficacious secondary and tertiary responses. At these times, additional vaccine would be released.



E263-051 50X

E263-051 1000X

E263-055 50X



E263-055 1000X

5948-52

Figure 26. Photomicrographs taken by scanning electron microscopy of JE vaccine microcapsules prepared with 50:50 DL-PLG (above) and 63:37 DL-PLG (below): Batches E263-051 and E263-055. These microcapsules were used in the initial in vivo immunization studies.

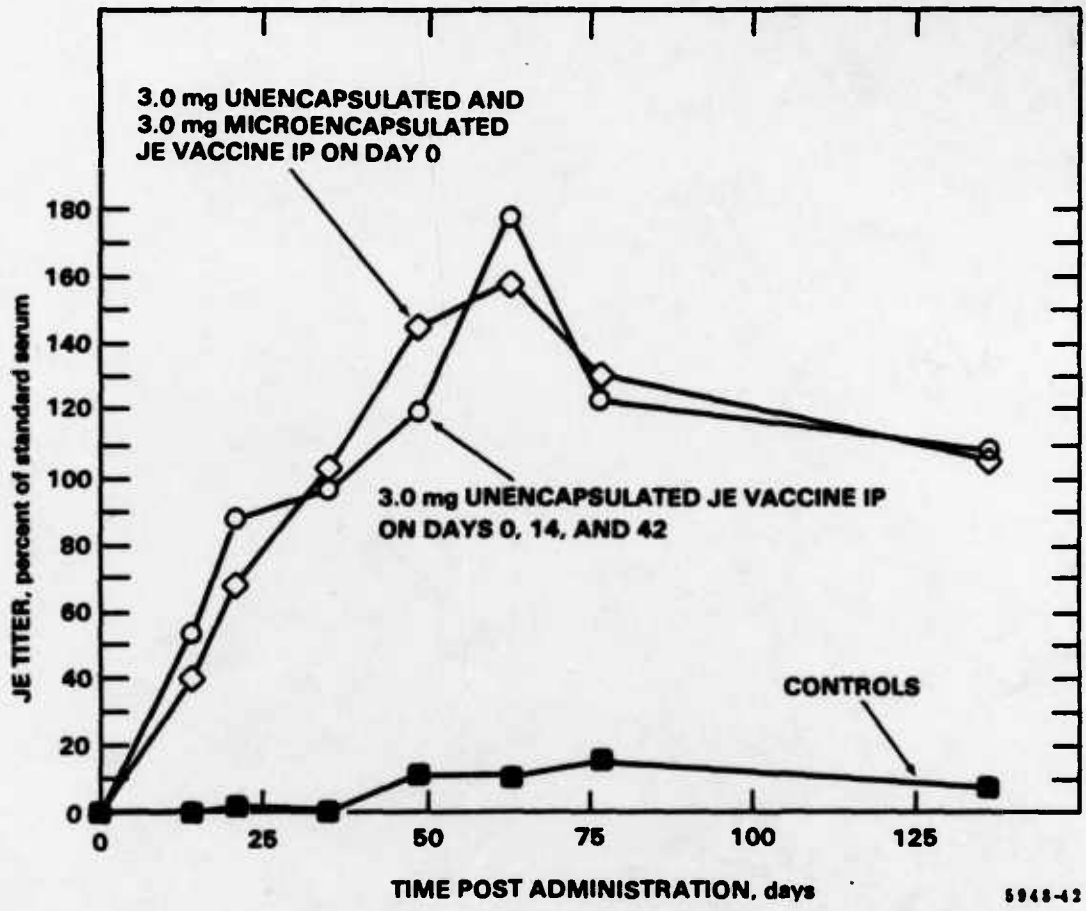


Figure 27. ELISA results obtained from immunization studies with a prototype JE vaccine microcapsule formulation.

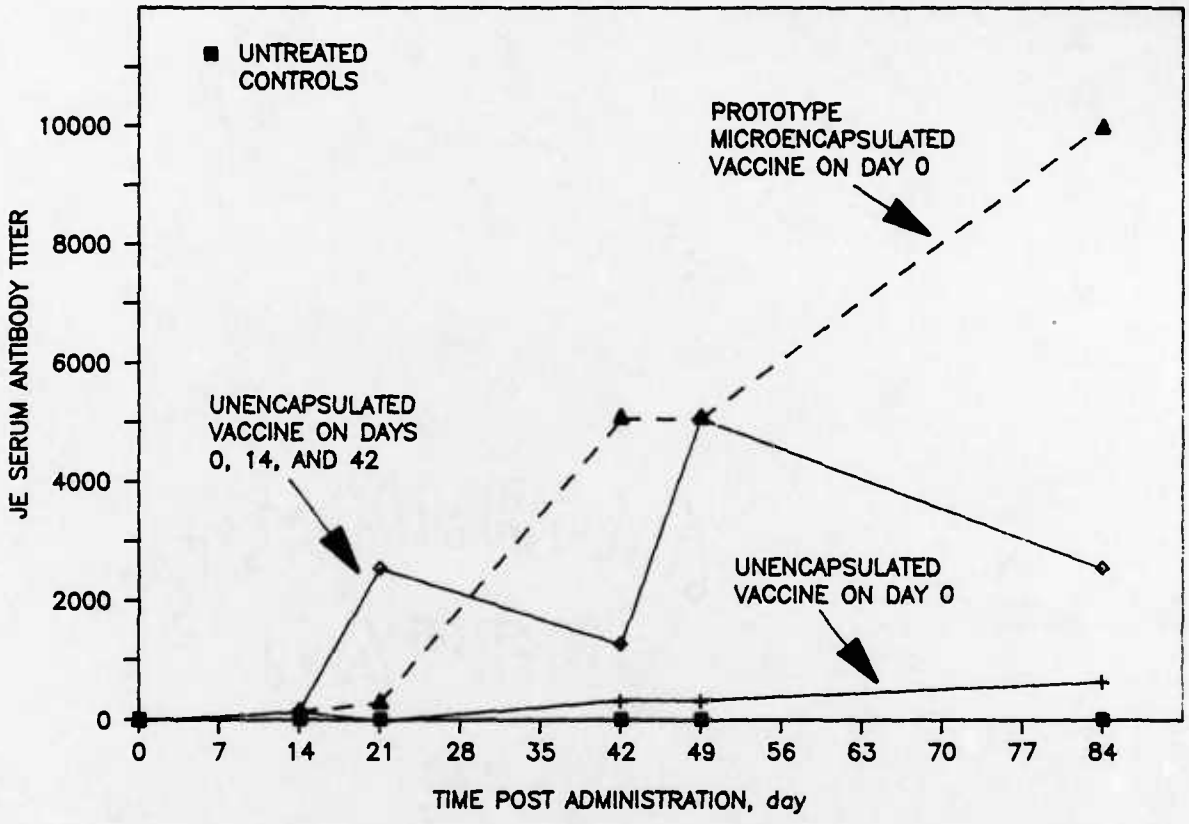


Figure 28. Neutralizing antibody titers from pooled serum of animals treated with various JE vaccine formulations: 50% plaque-reduction end point.

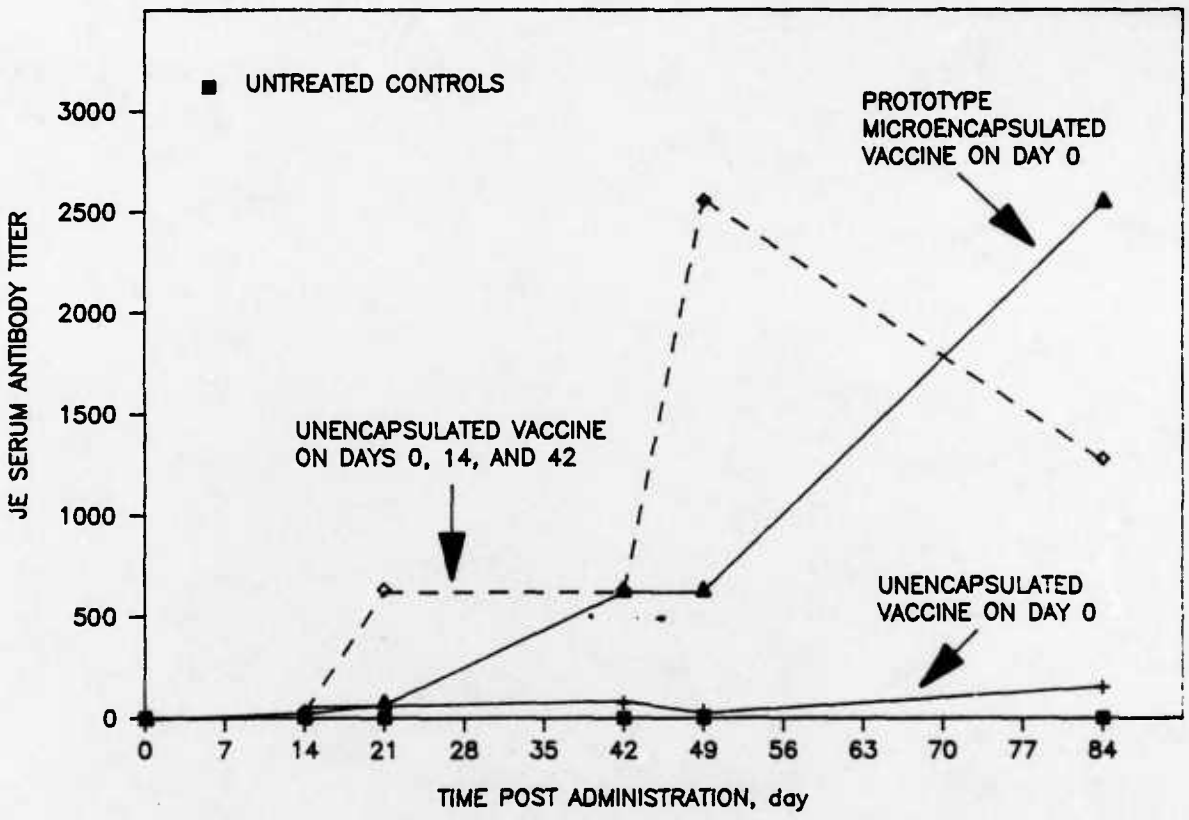
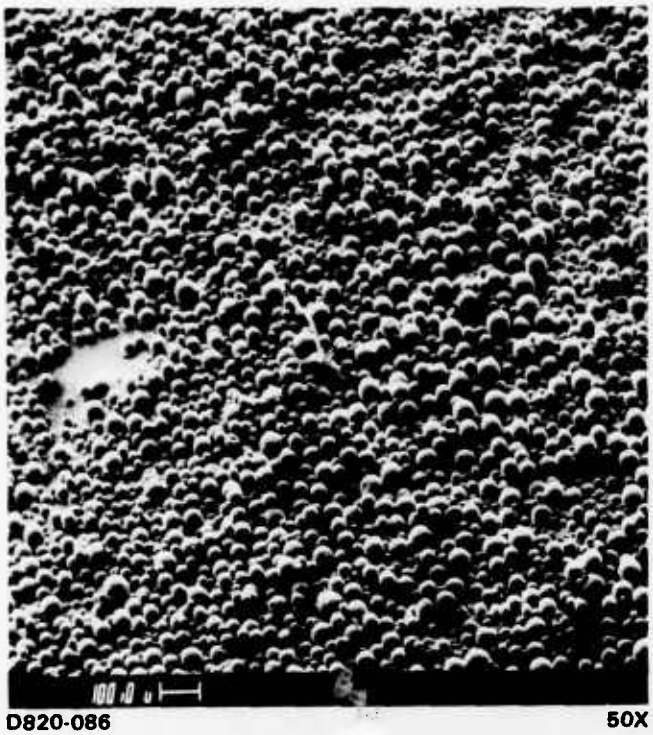
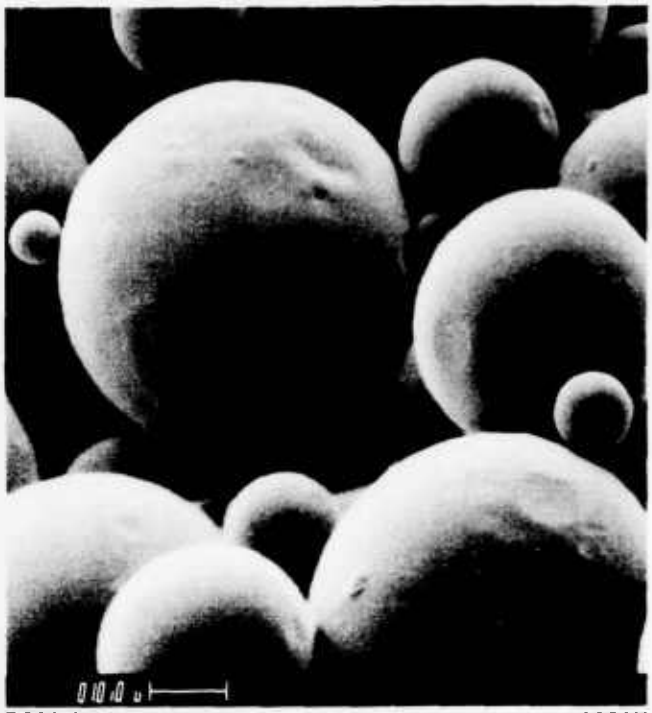


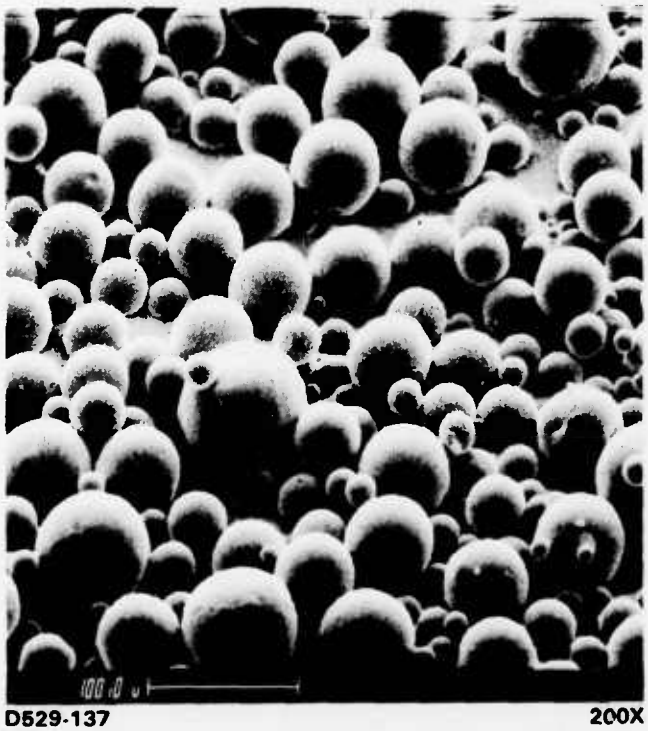
Figure 29. Neutralizing antibody titers from pooled serum of animals treated with various JE vaccine formulations: 80% plaque-reduction end point.



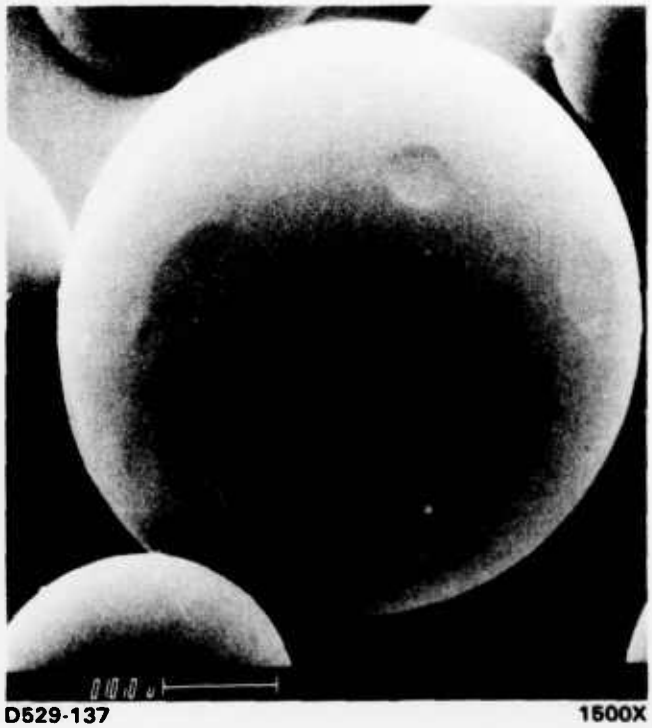
D820-086 50X



D820-086 1000X



D529-137 200X



D529-137 1500X

5948-88

Figure 30. Photomicrographs taken by scanning electron microscopy of muramyl tripeptide microcapsules prepared with a 50:50 DL-PLG excipient: Batches D820-086 and E529-137.

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