AD L (2)

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

Annual Report

Thomas R. Tice, Richard M. Gilley, K. Larry Pledger,
William M. Shannon, Melinda G. Hollingshead, Louise Westbrook

May 29, 1987

Supported by

US ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-86-C-6044

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

July 7, 1987
Project 5948-V1 (Annual)

UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Distribution limited to US Government Agencies only; Proprietary Information, July 7, 1987.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S) SoRI-EAS-87-678	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Southern Research Institute 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) 2000 Ninth Avenue South P.O. Box 55305 Birmingham, Alabama 35255-5305	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-86-C-6044
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 63763 / PROJECT NO. 3M1 63763D807 / TASK NO. AD / WORK UNIT ACCESSION NO. 053

11. TITLE (Include Security Classification)
ENHANCEMENT OF ANTIVIRAL AGENTS THROUGH THE USE OF CONTROLLED-RELEASE TECHNOLOGY (Unclassified)

12. PERSONAL AUTHOR(S)
Tice, T.R.; Gilley, R.M.; Pledger, K.L.; Shannon, W.M.; Hollingshead, M.G.; Westbrook, L.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 10/15/85 TO 1/31/87	87 May 29	43

16. SUPPLEMENTARY NOTATION

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	01	
06	15	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Controlled-release; Microcapsules; Drug delivery; Japanese encephalitis; (Poly I·C); Interferon

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

This is our First Annual Progress Report on Contract DAMD17-86-C-6044, entitled "Enhancement of Antiviral Agents through the use of Controlled-Release Technology." It covers research completed during the period from October 15, 1985, through January 31, 1987.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL Mary F. Bostian	22b. TELEPHONE (Include Area Code) 301-663-7325 / 22c. OFFICE SYMBOL SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete.*

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

Use or disclosure of data contained on this sheet is subject to the restrictions on the cover of this report.

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).

I. SUMMARY

This First Annual Progress Report on Contract DAMD17-86-C-6044, entitled "Enhancement of Antiviral Agents through the use of Controlled-Release Technology." It covers research completed during the period from November 1, 1985, through January 31, 1987.

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 the first year of our work, we have prepared and characterized four batches of poly(DL-lactide-co-glycolide) to be used as the polymeric excipients in the microencapsulation work. In addition, we have actively pursued development and testing of poly(I•C) and JE vaccine microcapsule formulations. Because most of our efforts were directed toward the development and testing of poly(I•C) and JE vaccine microcapsule formulations, we have not yet begun work with MTP or IFN.

II. INTRODUCTION

In today's battlefield environment, 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 from 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. One 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 released 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 environment 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 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 extended 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 the immune modulators and antiviral agents, muramyl tripeptide, interferon, and poly(I•C).

III. PREPARATION AND CHARACTERIZATION OF POLYMER EXCIPIENTS

During this reporting period, we synthesized and characterized four batches of polymer for use in our microencapsulation process work. More specifically, we prepared polymer excipients with lactide/glycolide mole ratios of 53:47, 66:34, 89:11, and 100:0. The general procedures that we used to prepare these polymers are outlined below.

A. DL-Lactide Purification

The DL-lactide used to prepare the polymer was purchased from Gallard-Schlesinger (Carle Place, NY). To purify the monomer, it is first dissolved by heating a mixture of the monomer in a volume of dry ethyl acetate (stored

over molecular sieves) about equal to its weight. While still hot, the solution is vacuum filtered through an extra coarse, sintered-glass gas-dispersion tube. The solvent level is reduced with an aspirator to a level equal to about half the weight of the lactide. The solution is then allowed to cool slowly to room temperature and chilled in an ice-water bath to effect crystallization. The monomer is finally filtered in a nitrogen-filled glove box. The monomer is recrystallized from ethyl acetate two additional times in this manner. All glassware used after the initial hot filtration and recrystallization is oven-dried overnight at 150 °C prior to use. After the final recrystallization, the purified monomer is vacuum-dried in a desiccator and stored in oven-dried glass jars until ready for use.

B. Glycolide Synthesis and Purification

The glycolide monomer is prepared and purified by the following method. Excess water is first distilled from 67% aqueous glycolic acid (Eastman Chemicals, Rochester, NY) in a 3-neck flask equipped with a thermometer, distillation head, and a condenser as shown in Figure 1. The solution is boiled at reduced pressure with the use of a water aspirator. After the excess water has evolved, heating is continued to remove additional water by dehydration of the glycolic acid. After no further water is evolved, the flask is allowed to cool to room temperature under vacuum. At this point, about 1 wt % antimony oxide, based on the theoretical glycolic acid content, is added to the flask as a catalyst. The distillation head and condenser are removed, and the flask is connected to two receiving flasks and a trap arranged in series as shown in Figure 2. The receiving flasks and trap are cooled by dry-ice/isopropanol baths. (Note: The first receiving flask is for product collection. The second receiving flask is actually a trap.) The pressure is reduced to about 2 mmHg, and the reaction flask is heated to distill the crude glycolide. The material that distills between 110 and 130 °C is collected in the first receiving flask.

The crude glycolide collected is next purified by first washing the product. This is achieved by slurrying the glycolide in isopropanol, followed by filtering and vacuum-drying, and then by three recrystallizations from ethyl acetate. After washing, precautions are made to protect the glycolide from atmospheric moisture during each stage of recrystallization by using oven-dried glassware, dry ethyl acetate (stored over molecular sieves), and a glove box filled with nitrogen. The crude glycolide is combined with a volume of ethyl acetate approximately equal to three-fourths its weight. And the mixture is then heated to reflux to dissolve the glycolide and cooled slowly to room temperature to allow crystallization. The monomer is recrystallized three times in this manner. After each recrystallization, the glycolide crystals are collected by vacuum filtration in a glove box. After the final recrystallization, the product is dried at room temperature under a vacuum of <2 mmHg in a desiccator. The purified dried monomer is then stored in oven-dried glass jars placed inside a desiccator.

C. Copolymer Synthesis

All glassware is oven dried at 150 °C overnight and allowed to cool in a nitrogen-filled glove box. All handling of the reactants and assembling of apparatus is done in the glove box. The purified monomers are weighed directly into a 3-neck, round-bottom flask as shown in Figure 3. The flask is then fitted with a mechanical stirrer and a gas-inlet tube. After being charged and sealed, the flask assembly is evacuated three times, back filled with nitrogen, removed from the glove box, connected to a dry nitrogen purge, and placed into an oil bath maintained at 170 °C. Once the monomers have partially melted, stirring is begun. Positive nitrogen pressure is maintained over the monomers throughout the polymerization. After the monomers have completely melted, 0.05 wt % of stannous octoate is introduced into the flask with a microsyringe. Stirring is continued until the mixture becomes too viscous to stir, at which point the stirrer is raised out of the melt. The polymerization is then continued for a total reaction time of 16 to 18 h. Next, the resulting polymer is allowed to cool to room temperature under a nitrogen atmosphere and removed by breaking the flask. Any residual glass is removed from the polymer plug by submerging it into liquid nitrogen. While cold, the polymer is broken into several smaller pieces and dissolved in methylene chloride and precipitated into methanol. The solvent is then removed by evaporation at room temperature under a hood and, finally, under vacuum at <2 mmHg and about 40 °C. The yields are typically about 75% of theoretical. The polymers are then characterized and stored in a desiccator until ready for use.

After synthesis and purification, we thoroughly characterized the polymer excipients. More specifically, we determined the lactide/glycolide mole ratio by nuclear magnetic resonance (NMR) spectroscopy, the inherent viscosity by dilute-solution viscometry, and the polymer molecular weights by gel-permeation chromatography. The characterization data we obtained for each of the polymer excipients is summarized in Table 1.

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

A. Preparation of Poly(I•C) Microcapsules

During the course of the first year of the project, significant emphasis was directed toward developmental work with poly(I•C). The initial phase of our work involved development of analytical procedures to determine the core loadings (drug content) and the in vitro release kinetics of the microcapsules. After development of suitable analytical procedures, the microencapsulation process work was initiated. Several microencapsulation processes were compared and tested to ensure that the poly(I•C) retained its activity during the micro-

encapsulation processing. Poly(I•C) is a double-stranded molecule, and it is imperative that its double-strand configuration not be altered. If altered, the desired biological response will be lost.

Many batches of microcapsules were prepared in an effort to identify the optimum product. After preparation, each batch of microcapsules was thoroughly characterized. This characterization included examination of the microcapsule surface morphology by scanning electron microscopy (SEM) and determination of the core loading. We also determined how well the poly(I•C) was encapsulated using a short-term in vitro release analysis.

After we identified poly(I•C) microcapsule formulations which exhibited promising results in vitro, samples of these microcapsule formulations were sent to USAMRIID for in vivo efficacy testing. In the tests performed at USAMRIID, the microcapsules were administered to experimental animals (mice) which were subsequently challenged with Rift Valley Fever virus. The survival rates of the animals were monitored and compared with the untreated animals to determine the efficacy of the microcapsule formulations. The results of these initial studies were encouraging, however optimization of the microcapsule formulation will be necessary to obtain an injectable formulation capable of providing complete protection against viral challenge in the test animals.

B. Characterization of Poly(I•C) Microcapsules

1. Surface morphology

We examined the surface morphology of poly(I•C) microcapsules from photomicrographs taken by scanning electron microscopy (SEM). Figure 4 shows photomicrographs of representative batches of poly(I•C) microcapsules. As can be seen, these microcapsules have smooth, continuous DL-PLG coatings with no evidence of unencapsulated poly(I•C) on their surfaces. Such a uniform coating is essential to achieve the desired release of poly(I•C) from the microcapsules.

2. Core loading and encapsulation efficiency

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

A known weight of microcapsules is added in methylene chloride which dissolves the DL-PLG. The undissolved poly(I•C) core material is then extracted into an aqueous 0.15 M sodium chloride solution which is quantified for poly(I•C) spectrophotometrically at 250 nm. The theoretical core loading for a batch of microcapsules is based upon the copolymer and poly(I•C) input and is calculated in the following manner:

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

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:

$$\text{Actual core loading, wt \%} = \frac{\text{Poly(I•C) quantified, g}}{\text{Microcapsules used in assay, g}} \times 100 \text{ wt \%}$$

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

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

3. In vitro release determinations

We determined the release kinetics of the poly(I•C) microcapsules using a short-term in vitro procedure. The purpose of this short-term in vitro release analysis is to assess whether the microcapsules will "dump," that is, release a major amount of their poly(I•C) at an uncontrolled rate. This analysis can also be called a "burst test".

Briefly stated, the analysis involves placing a known weight of microcapsules into a receiving fluid consisting of aqueous 0.15 M sodium chloride solution. This solution is placed in a shaker bath maintained at 37 °C. Aliquots are then periodically removed from the receiving fluid and quantified for poly(I•C) spectrophotometrically. The aliquots are then returned to the receiving fluid.

Table 2 lists the in vitro release data we obtained for each significant batch of poly(I•C) microcapsules that we prepared. It is important to note that the in vitro release data by no means indicates the actual amount of poly(I•C) that will be released from the microcapsules when tested in vivo. Rather, this release procedure is used to test for burst effects and for comparison of microcapsules from different batches.

V. RESULTS OF ANIMAL STUDIES WITH POLY(I•C) MICROCAPSULES

During the past year, we sent 16 different poly(I•C) microcapsule formulations to USAMRIID for Rift Valley Fever virus challenge experiments in mice. These microcapsule formulations exhibited different in vitro release rates and were made for experiments designed to correlate in vitro release profiles with the survival of test animals challenged with Rift Valley Fever virus. In the survival experiments conducted at USAMRIID, the test mice were injected i.p. with the poly(I•C) microcapsule formulations on Day -1 of the study. The mice were then exposed to Rift Valley Fever virus on Day 0. The mice receiving the microcapsules as well as untreated controls were then monitored for survival over the next 18 days.

No deductions could be determined from some of the earliest viral-challenge experiments because the untreated control animals survived. From the results of the last six formulations tested, however, several conclusions can be drawn. The cumulative in vitro release profiles for these formulations are shown in Figures 5 and 6 while the survival rate of mice treated with these formulations and the daily release rates are shown in Figures 7 through 12. Examination of the cumulative in vitro release profiles reveals that with the exception of Batch E100-020, all of the formulations show very little release of poly(I•C) after Day 2. Batch E100-020 releases poly(I•C) at a fairly constant rate for 11 days.

Examination of the survival curves obtained with these formulations shows that the survival rates varied from 0 to 90%. Comparison of the survival curves and the daily in vitro release profiles of each formulation shows that the survival rate of the test animals was higher with the formulations that released poly(I•C) for 7 to 10 days. The best survival rate (90%) was obtained with Batch E100-020. As can be seen in Figure 12, this formulation demonstrated the best controlled release--about 40 to 60 µg of poly(I•C) was released per day for 11 days. The poorest survival rate obtained (0%) was with Batch D763-013. As shown in Figure 7, this formulation released almost all of the poly(I•C) the first day with no additional release after Day 2. The results of these experiments indicate that the most efficacious poly(I•C) microcapsule formulations are those that demonstrate a continuous controlled release of poly(I•C) for 10 to 14 days.

In addition to the viral challenge experiments, we conducted pharmacodynamics studies on two of the poly(I•C) microcapsule formulations--Batches E100-019 and -020. The pharmacodynamic studies involved administering the microcapsules to animals and measuring interferon levels in the serum of the animals. The experimental procedures that we used to conduct the studies are outlined below.

The test animals were four-week-old female CD-1 Swiss mice (VAF Plus) obtained from Charles River Laboratories. After quarantine, the mice were divided into five groups of 30, designated as Groups 1 through 5, and marked with an ear punch so that each individual mouse could be distinguished. Because each mouse could not be bled on a daily basis, the 30 mice in each group were further subdivided into six subgroups for bleeding purposes, designated Subgroups A through F. Subgroups A and B were bled on Days -1, 2, and 5; Subgroups C and D on Days 0, 3, and 6; Subgroups E and F on Days 1, 4, and 7.

The mice in Group 1 received a single i.p. injection on Day 0 of 170 mg of placebo microcapsules in 1.5 mL of injection vehicle (2 wt % carboxymethyl cellulose and 1 wt % Tween 20 in water). Group 2 received a single i.p. injection of 200 µg of encapsulated poly(I•C) (Formulation E100-019) in 1 mL of vehicle. Group 3 received a single i.p. injection of 500 µg of encapsulated poly(I•C) (Formulation E100-020) in 1.5 mL of vehicle. (Note: Different doses of microencapsulated poly(I•C) were used so that the amount of poly(I•C) released each day would not exceed about 100 µg.) Group 4 received i.p. injections of 50 µg of unencapsulated poly(I•C) on Days 0, 1, and 4. Group 5 consisted of normal, untreated control animals.

To obtain base-line interferon levels, serum samples were collected on Day -1 from 10 mice from each of the groups. The next serum collection was on Day 0 one hour after we injected the mice with the microcapsules and the control articles. Serum samples were taken daily through Day 7 as described above.

The blood was obtained by bleeding the mice from the retro-orbital sinus into heparinized microhematocrit tubes. The blood was transferred to 250-µL microfuge tubes and held on ice until the tubes were centrifuged at 5000 rpm for five minutes in a Savant microfuge (Savant Inc., Farmingdale, NY). The serum was removed and stored at -20 °C until quantified for interferon.

To measure the interferon levels in the serum samples, vesticular stomatitis virus (VSV) was used in L929 cells in a cytopathogenic inhibition (CPE) assay. The serum samples were diluted in serial two-fold dilutions in Eagle's minimum essential medium supplemented with 5% heat-inactivated fetal bovine serum and added to L929 cells pregrown in 96-well microtiter plates. The test samples (0.1 mL per well) were incubated with the cells for six hours before the addition of 0.1 mL of of a dilution of stock VSV, which provided an inoculum of 32 cell culture infectious dose 50% ($CCID_{50}$/0.1 mL). The plates were sealed and incubated at 37 °C for three days. Then, each well was examined microscopically and the degree of CPE scored. The interferon titer was read as the reciprocal of the dilution that protected approximately 50% of the cells from virus-induced CPE.

The amount of poly(I•C) released from each microcapsule formulation in vitro and the corresponding interferon titer that was observed in the test

animals are shown in Figures 13 and 14. With both formulations, the mice showed high titers of interferon the day after the microcapsules were injected, especially the animals that received Batch E100-019. Approximately two days after the injection, however, the interferon titers of the test animals were minimal. Similar results were obtained with the control animals that were administered 50 µg of unencapsulated poly(I•C) on Days 0, 1, and 4 of the study.

Although neither of the microcapsule formulations resulted in elevated interferon titers past Day 1 of the study, the results of the Rift Valley Fever virus challenge experiments with these formulations showed very contrasting results--Batch E100-020 resulted in a 90% survival rate whereas Batch E100-019 only had a 20% survival rate. These results indicate that poly(I•C) might be protecting the test animals by some mechanism other than the induction of interferon.

VI. PREPARATION AND CHARACTERIZATION OF JE VACCINE MICROCAPSULES

A. Preparation 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 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 two-to three-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. These 50:50 DL-PLG microcapsules will elicit the secondary response. After a second dormancy period, the microcapsules prepared with 65:35 to 85:15 DL-PLG will biodegrade and release JE vaccine. This third pulsatile release will occur about two months after the microcapsules are administered and will elicit the tertiary response. This programmed release of JE vaccine will allow complete immunization with a single injection.

After we developed analytical procedures for characterizing JE vaccine microcapsules and performed solvent compatibility tests, microencapsulation process work was initiated. Numerous batches of JE vaccine microcapsules were prepared by several different microencapsulation processes. After preparation, each batch of JE vaccine microcapsules was thoroughly characterized. This characterization included examination of the microcapsule surface morphology by SEM and determination of the core loading and in vitro release kinetics. Figure 15 shows photomicrographs taken by SEM of representative batches of

prototype JE vaccine microcapsules and Table 3 summarizes the characterization data for each batch of JE vaccine microcapsules prepared during the first year of the contract.

B. Solvent-Stability Testing of JE Vaccine

Prior to starting our encapsulation work, we subjected the JE vaccine to four solvents that we commonly employ in our microencapsulation processes. To accomplish this solvent-stability study, approximately 10 mg of JE vaccine (Biken, Lot Number 58-6) was placed in each of four test tubes. Next, 5 mL of solvent was added to each tube. The test tubes were then agitated for about 1 min and placed under vacuum to evaporate the solvent. Also, JE vaccine was subjected to the same four solvents while we homogenized the vaccine. We then measured by an enzyme-linked immunosorbent assay (ELISA) the various samples of solvent-treated JE vaccine we had prepared to determine if the microencapsulation solvents and/or procedures denatured the JE vaccine and thus altered its antigenicity.

The test we performed was as follows: An ELISA was developed for the detection of JE vaccine antigen. For this assay we used 96-well enzyme immunosorbent assay plates coated with antigen (solvent-treated JE vaccine). We then incubated the plates with antibody (immune serum obtained from C57GL/6 mice immunized with JE vaccine), followed by peroxidase-conjugated antimouse IgG, and finally ABTS [2,2'-azino-di-(3-ethylbenzthiazoline sulfonate)] substrate. The optical density of each well was read at 405 nm on an ELISA reader.

Five concentrations (300, 200, 100, 50, and 25 µg/mL) of untreated and solvent-treated antigen were tested against a single antibody concentration (1:2000) that was known to produce a response ranging from positive to negative with the concentrations of JE vaccine antigen used. The optical-density readings we obtained are recorded in Table 4. As can be seen, the JE vaccine was significantly inactivated following homogenization in Solvents 1 and 4. On the other hand, there was no significant loss of the antigenic properties of the JE vaccine in Solvents 2 and 3. We used these latter two solvents in the microencapsulation work with the JE vaccine.

C. Characterization of JE Vaccine Microcapsules

The second phase of work with JE vaccine involved development of analytical procedures to determine the core loading and in vitro release kinetics of the microcapsule formulations. Several complications were encountered in developing these procedures, but assays which gave reproducible results have been developed. The procedures we use to characterize the JE vaccine microcapsules we prepared are described below.

1. Surface morphology

We examined the surface morphology of JE vaccine microcapsules from photomicrographs taken by SEM. Figure 15 shows photomicrographs of representative batches of JE vaccine microcapsules. As can be seen, these microcapsules are relatively spherical particles with no evidence of unencapsulated JE vaccine on their surfaces. Although pin holes are evidenced on the surface of the microcapsules, in vitro release data for these microcapsules indicate that the holes are only at the surface and do not penetrate into the microcapsules.

2. Core loading and encapsulation efficiency

The core loading of the JE vaccine microcapsules is a measure of the amount of JE vaccine incorporated inside the microcapsules. Our core-loading determination is based on the extraction of JE vaccine (core material) from a known amount of microcapsules and quantification of the extracted JE vaccine by using a UV spectrophotometric assay. The procedure we use follows.

A known weight of microcapsules is dissolved in chloroform. (The amount of microcapsules to be used in the core-loading determination depends on the theoretical core loading of the microcapsules. The amount of microcapsules should contain a sufficient quantity of drug to fall in the upper portion of the standard curve.) The JE vaccine core material is then extracted into Nanopure water (water purified by a Nanopure II reverse-osmosis/deionization system, Sybron-Barnstead, Boston, MA) and purged with nitrogen to remove all trace amounts of chloroform. The sample is then quantified for JE vaccine spectrophotometrically at 200 nm.

The actual core loading is determined by quantifying the JE vaccine content of the microcapsules by using the procedure described above. The actual core loading is calculated in the following manner:

$$\text{Actual core loading, wt \%} = \frac{\text{Amount of JE vaccine in the microcapsules, g}}{\text{Total amount of microcapsules used in assay, g}} \times 100 \text{ wt \%}$$

The theoretical core loading for a batch of microcapsules is based upon the DL-PLG and JE vaccine input and is calculated in the following manner:

$$\text{Theoretical core loading, wt \%} = \frac{\text{JE vaccine input, g}}{\text{(DL-PLG input, g) + (JE vaccine input, g)}} \times 100 \text{ wt \%}$$

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

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

3. Determination of in vitro release kinetics

We determined the release kinetics of the JE vaccine microcapsules using a short-term in vitro analysis. The purpose of this short-term in vitro release analysis is to assess whether the microcapsules will release a major amount of their JE vaccine at an uncontrolled rate. (Note: If all of the JE vaccine is released early on, none will be available for the secondary release.)

Briefly stated, our short-term in vitro release analysis involves placing a known weight of microcapsules into a receiving fluid consisting of 4 mL of Nanopure water. (Again, an appropriate amount of microcapsules should be used, i.e., an amount such that a release of 5 to 100% of the JE vaccine contained within the microcapsules will fall within the range of the standard curve.) The receiving fluid is then placed in an incubator maintained at 37 °C. The receiving fluid is periodically removed and quantified for JE vaccine spectrophotometrically. Fresh receiving fluid is then put into the container with the test microcapsules to maintain a constant volume throughout the release study.

VII. ANIMAL EXPERIMENTS WITH UNENCAPSULATED JE VACCINE

During the first year of this contract, no experiments were conducted with microencapsulated JE vaccine. These experiments have been delayed due to the failure of required personnel to develop a suitable antibody titer after several injections with JE vaccine. However, JE vaccine microcapsules have been prepared for administration to experimental animals and these experiments will begin at the beginning of Year 2 of the contract. After the initial prototype formulations are evaluated, optimization of the formulation will proceed.

Experiments with unencapsulated JE vaccine have also begun to ensure that the vaccine, supplied to us by BIKEN, would induce the desired immunonological effect in animals.

VIII. ACKNOWLEDGMENTS

Mr. James P. English, Head of the Polymer Engineering Section, and Phillip E. Arnold, Research Chemical Technician, prepared the polymer excipients. Mr. David A. Dunshee, Assistant Chemist, Ms. Elvera A. Thomason, Research Chemical Technician, Ms. Lisa D. Swift, Chemical Technician, and Ms. Elizabeth E. Edmonson, Chemical Technician, prepared and analyzed the poly(I•C) and JE vaccine microcapsules. Ms. Louise Westbrook, Research Biologist, Ms. Jeanine Qualls, Assistant Biologist, and Ms. Ellen S. Stringfellow, Assistant Biologist, performed ELISA assays on solvent-exposed JE vaccine and performed animal experiments to determine the interferon levels of mice treated with poly(I•C) microcapsules. In addition, Dr. Lorraine V. Brando, Research Virologist and Dr. Melinda G. Hollingshead, Research Immunologist, performed assays on and conducted the in vivo experiments with the JE vaccine.

Submitted by:

K. Larry Pledger
Associate Chemist

Richard M. Gilley
Head, Drug Delivery Section

Reviewed by:

Donald L. Hill for
William M. Shannon, Head
Microbiology-Virology Division
Biochemistry Department

Approved by:

Thomas R. Tice
Head, Microencapsulation Division

SoRI-EAS-87-678
July 7, 1987
Project 5948-VI (Annual)
NB: D574, D763, D402,
E100, E111, E202
(4:1::15) cob, kbm

APPENDIX A

TABLES

TABLE 1. PROPERTIES OF POLYMER EXCIPIENTS PREPARED FOR MICRCENCAPSULATION WORK

Excipient Batch	Lactide: glycolide,[a] mole ratio	Inherent viscosity,[b] dL/g	M_n,[c] daltons	M_w,[d] daltons	MWD[e]
D574-035-1	53:47	0.83	39,600	85,100	2.15
D762-008-1	66:34	0.89	71,200	82,300	1.16
D574-143-1	89:11	1.02[f]	62,200	120,900	1.94
D763-037-1	100:00	0.73[f]	95,500	127,300	1.33

[a] Lactide:glycolide mole ratio determined by NMR spectroscopy.

[b] Inherent viscosity determined with a Cannon-Fenske viscometer in hexafluorisopropanol at 30 °C and a concentration of about 0.5 g/dL.

[c] M_n = number-average molecular weight.

[d] M_w = weight-average molecular weight.

[e] MWD = molecular-weight distribution = M_w/M_n.

[f] Inherent viscosity determined in chloroform.

TABLE 2. PROPERTIES OF POLY(I·C) MICROCAPSULES PREPARED WITH DL-PLG EXCIPIENTS

Batch	Yield, % of theoretical	Core loading, wt % poly(I·C)		Encapsulation efficiency, % of theoretical	Poly(I·C) released in vitro, %, at				
		Theoretical	Actual		1 h	3 h	6 h	24 h	48 h
D574-063	70.9	1.00	1.18	118.0	10.2	17.9	--[a]	63.3	73.2
D574-069	81.7	0.57	0.34	59.6	6.1	18.7	55.3	100.0	--
D574-075	79.7	0.98	0.53	54.1	6.4	14.8	41.4	82.3	89.7
D574-085	77.9	0.91	0.74	81.3	--	13.9	26.8	57.6	63.3
D574-095	81.9	0.91	0.86	94.5	6.0	17.4	46.0	79.4	--
D574-101	40.5	0.90	0.33	36.6	4.2	10.3	22.7	57.1	72.5
D574-113	91.3	1.11	0.62	55.9	3.7	14.6	31.7	75.7	90.0
D574-147	61.2	1.04	0.86	82.7	1.4	1.6	14.8	41.9	48.9
D574-152	98.4	0.93	0.80	86.0	1.8	4.4	17.4	46.2	54.4
D574-160	97.4	0.93	0.75	80.6	4.7	28.1	52.3	100.0	--
D763-013	79.7	1.07	0.86	80.4	4.0	15.3	38.8	88.6	100.0
D763-016	97.8	1.08	0.80	74.1	9.1	25.3	44.0	76.1	91.4
D763-019	100.0	1.08	0.55	50.9	6.7	17.9	31.7	53.6	73.4
D763-013	79.7	1.07	0.86	80.4	4.0	15.3	38.8	88.6	100.0
D763-117	90.4	0.48	0.20	41.7	12.2	23.2	37.3	53.7	60.7
D763-121	87.8	0.48	0.25	52.1	2.8	7.6	12.3	20.5	26.9
D763-125	100.9	0.49	0.21	42.9	5.2	12.3	19.7	28.8	34.1
E100-019	101.8	0.93	0.80	86.0	2.4	17.2	40.9	58.9	75.8
E100-020	97.1	0.49	0.25	51.0	0.8	2.7	--	15.5	27.2

TABLE 3. PROPERTIES OF JE VACCINE MICROCAPSULES PREPARED WITH DL-PLG EXCIPIENTS

Microcapsule Batch	Yield, % of theoretical	Core loading, wt %		Encapsulation efficiency, % of theoretical
		Theoretical	Actual	
D902-013	83.1	2.36	2.6	110.2
D902-016	80.2	3.05	3.2	104.9
D902-018	87.5	2.45	3.6	146.9
D902-026	63.7	1.04	0.8	76.9
D902-027	76.0	1.02	ND[a]	ND
D902-039	74.2	1.17	1.3	111.1
D902-076	77.0	2.01	2.4	119.4
D902-078	75.3	4.84	5.5	113.6
D902-079	82.3	1.91	2.2	115.2
D902-081	79.7	2.15	1.9	88.4
D902-083	69.2	1.00	ND	ND
D902-084	63.3	4.51	4.0	88.7
D902-085	64.8	2.41	2.3	95.4
D902-103	59.3	5.02	3.2	63.7
D902-119	72.0	4.92	1.1	22.4
E100-035	52.9	1.42	1.6	112.7
E100-041	45.8	1.12	0.5	44.6
E100-043	30.8	1.35	0.4	29.6
E100-045	72.9	1.18	1.7	144.1
E100-047	74.6	1.02	1.9	186.3

[a]ND = Not determined.

TABLE 4. ELISA OPTICAL-DENSITY READINGS FOR JE VACCINE EXPOSED TO FOUR MICROENCAPSULATION SOLVENTS

Test sample	Solvent treatment	Homogenized JE vaccine	Antigen concentration, µg/mL					
			300	200	100	50	25	0
JE vaccine	No solvent	No	0.174	0.142	0.113	0.097	0.084	0.076
D902-6-1	Solvent 1	No	0.147	0.112	0.106	0.087	0.069	0.087
D902-6-2	Solvent 2	No	0.121	0.097	0.103	0.109	0.082	0.066
D902-6-3	Solvent 3	No	0.142	0.123	0.099	0.103	0.123	0.073
D902-6-4	Solvent 4	No	0.154	0.133	0.106	0.109	0.082	0.078
D902-7-1	Solvent 1	Yes	0.096	0.084	0.093	0.074	0.065	0.066
D902-7-2	Solvent 2	Yes	0.142	0.162	0.095	0.084	0.080	0.075
D902-7-3	Solvent 3	Yes	0.135	0.115	0.104	0.083	0.067	0.077
D902-7-4	Solvent 4	Yes	0.091	0.074	0.102	0.095	0.069	0.083

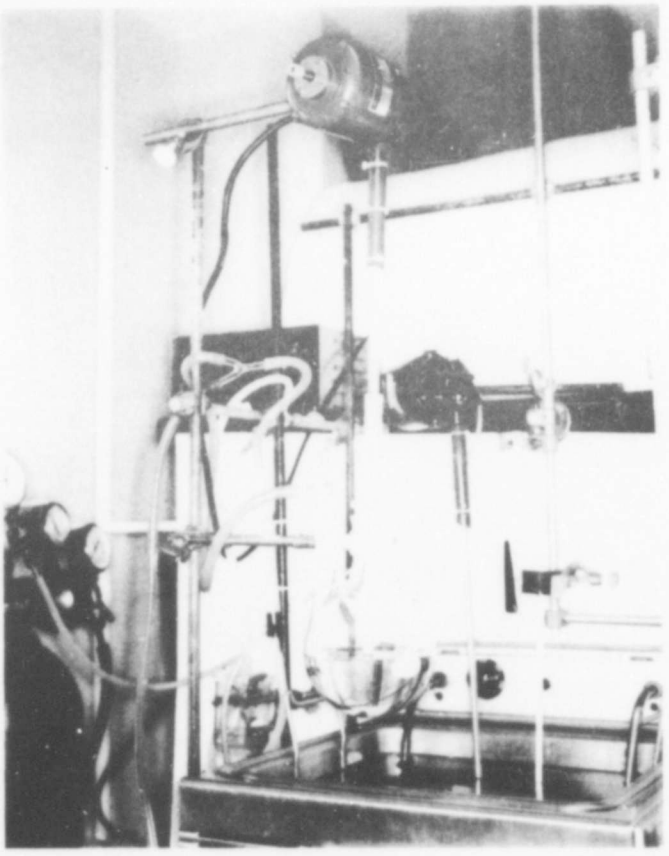
APPENDIX B

FIGURES

5256-1

Figure 1. Removal of excess water from glycolic acid.

Figure 2. Thermal cracking of dehydrated glycolic acid.



5256-3

Figure 3. Polymerization of lactide/glycolide copolymers.

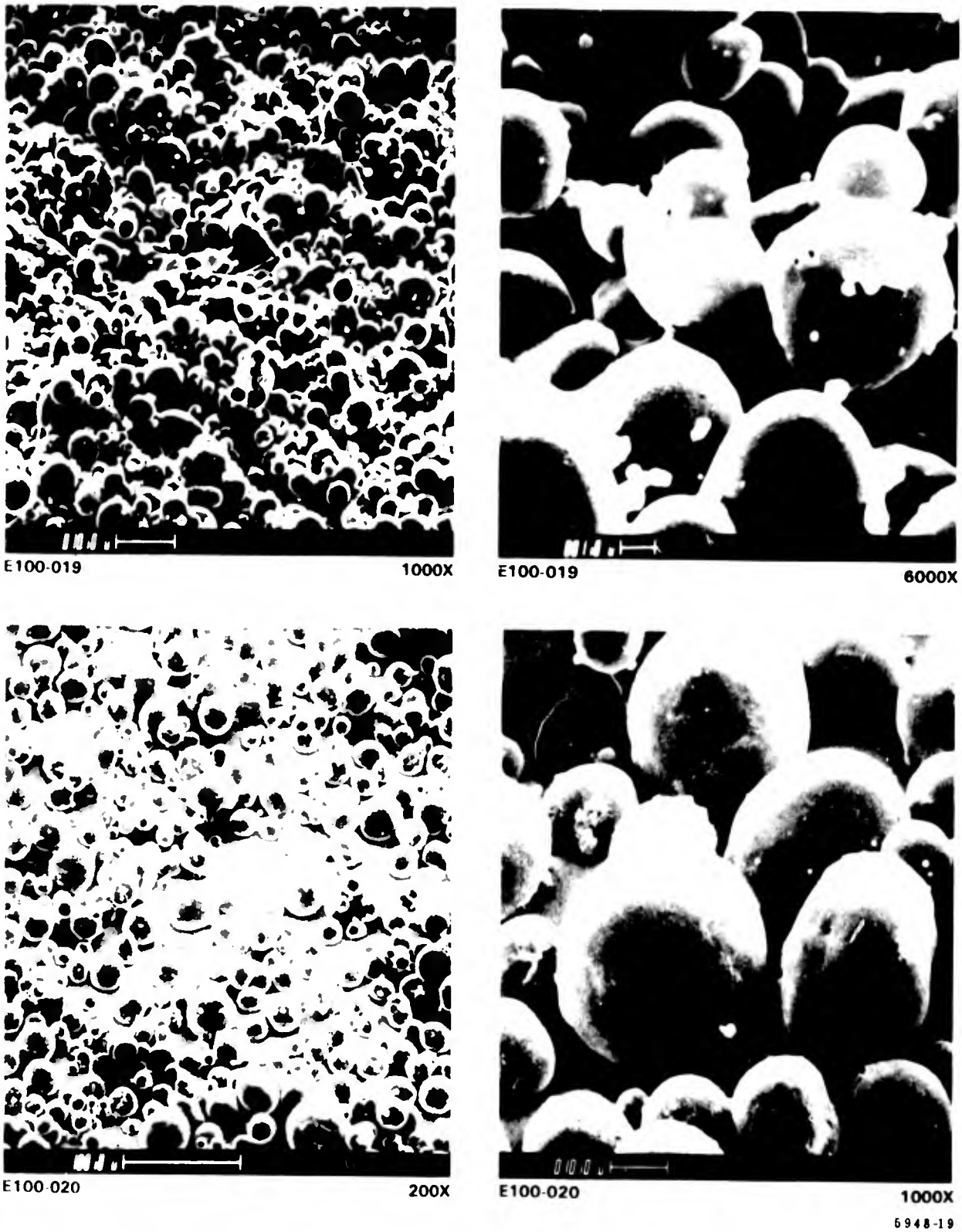


Figure 4. Photomicrographs taken by scanning electron microscopy of prototype poly(I·C) microcapsules prepared with a DL-PLG excipient: Batches E100-019 and E100-020.

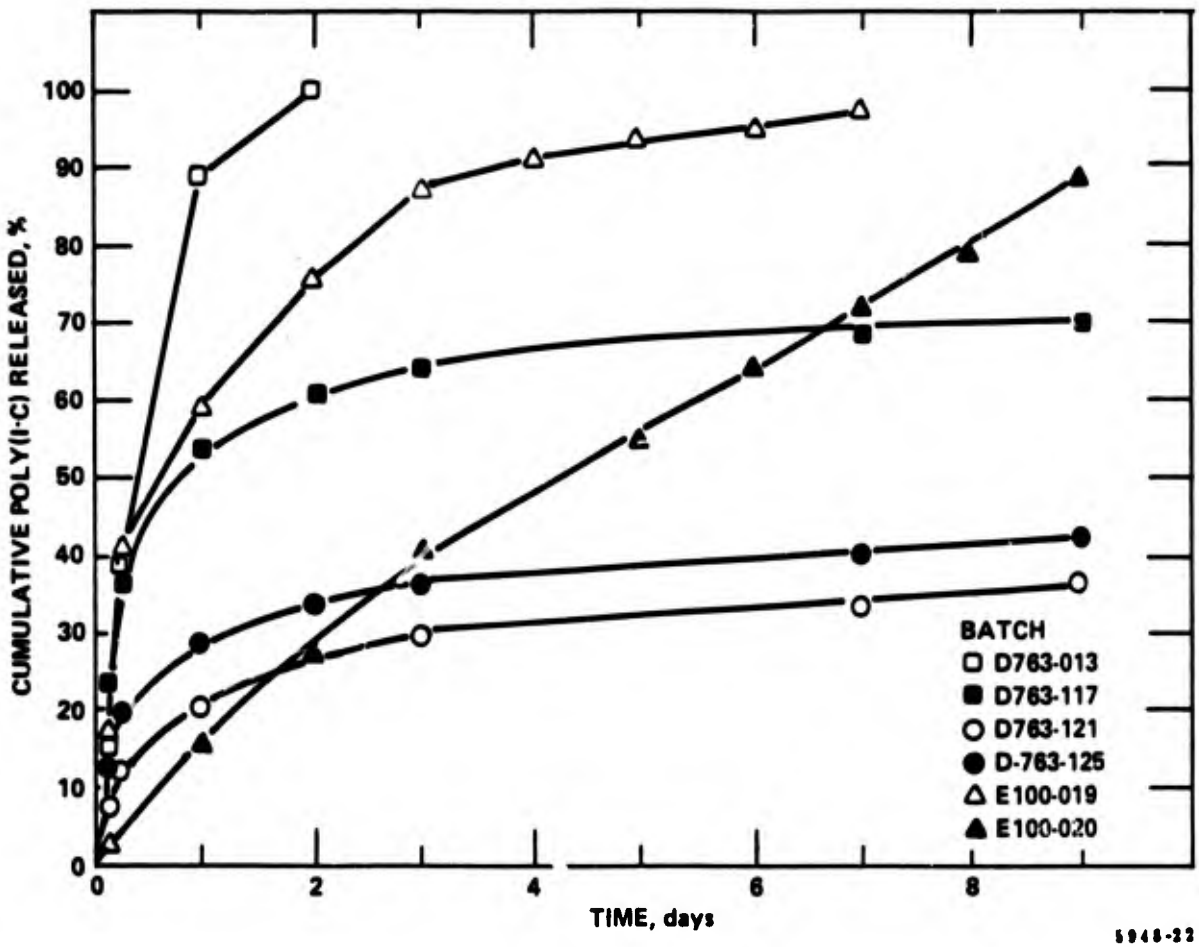


Figure 5. In vitro release profiles of poly(I·C) from microcapsules into a receiving fluid consisting of 0.15 M sodium chloride maintained at 37 °C. All of these microcapsule formulations were tested in Rift Valley Fever virus challenge experiments.

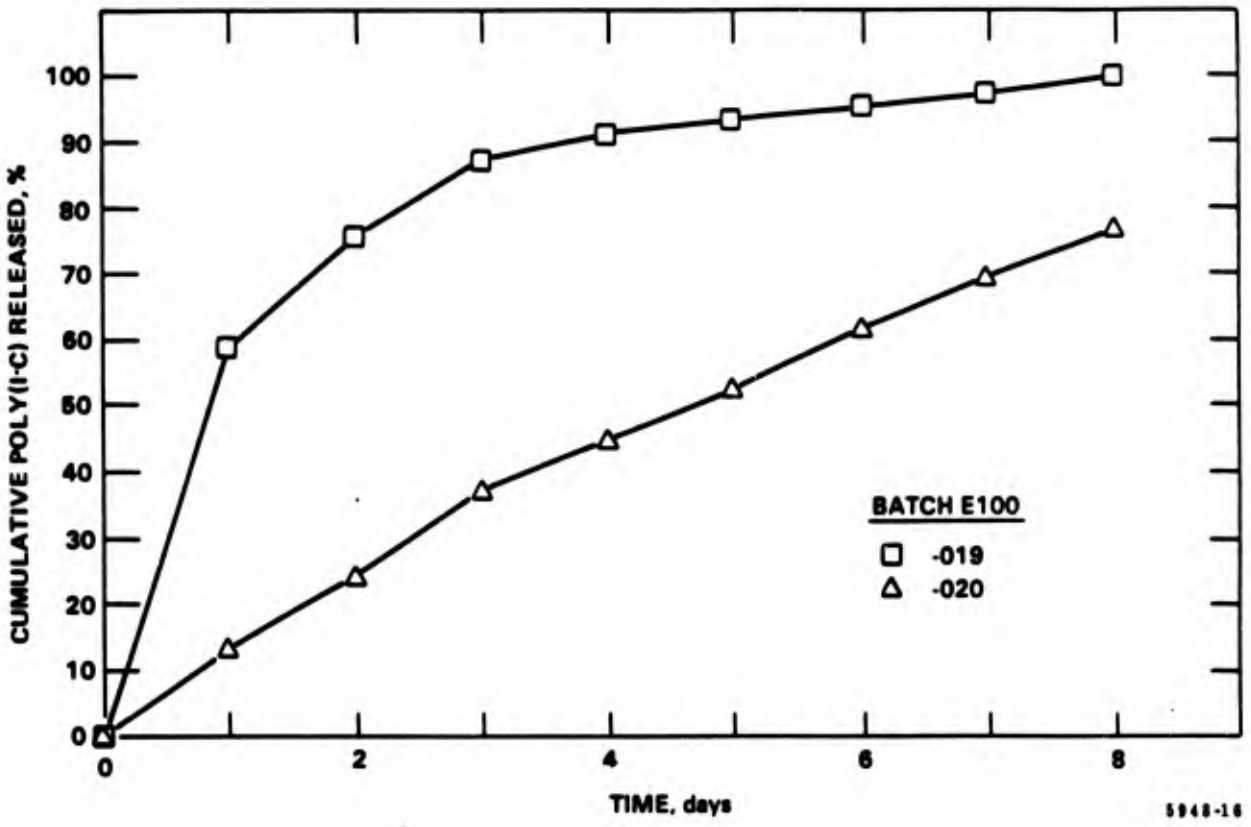


Figure 6. In vitro release kinetics of poly(I·C) from microcapsules into a receiving fluid consisting of 0.15 M aqueous sodium chloride maintained at 37 °C: Batches E100-019 and E100-020.

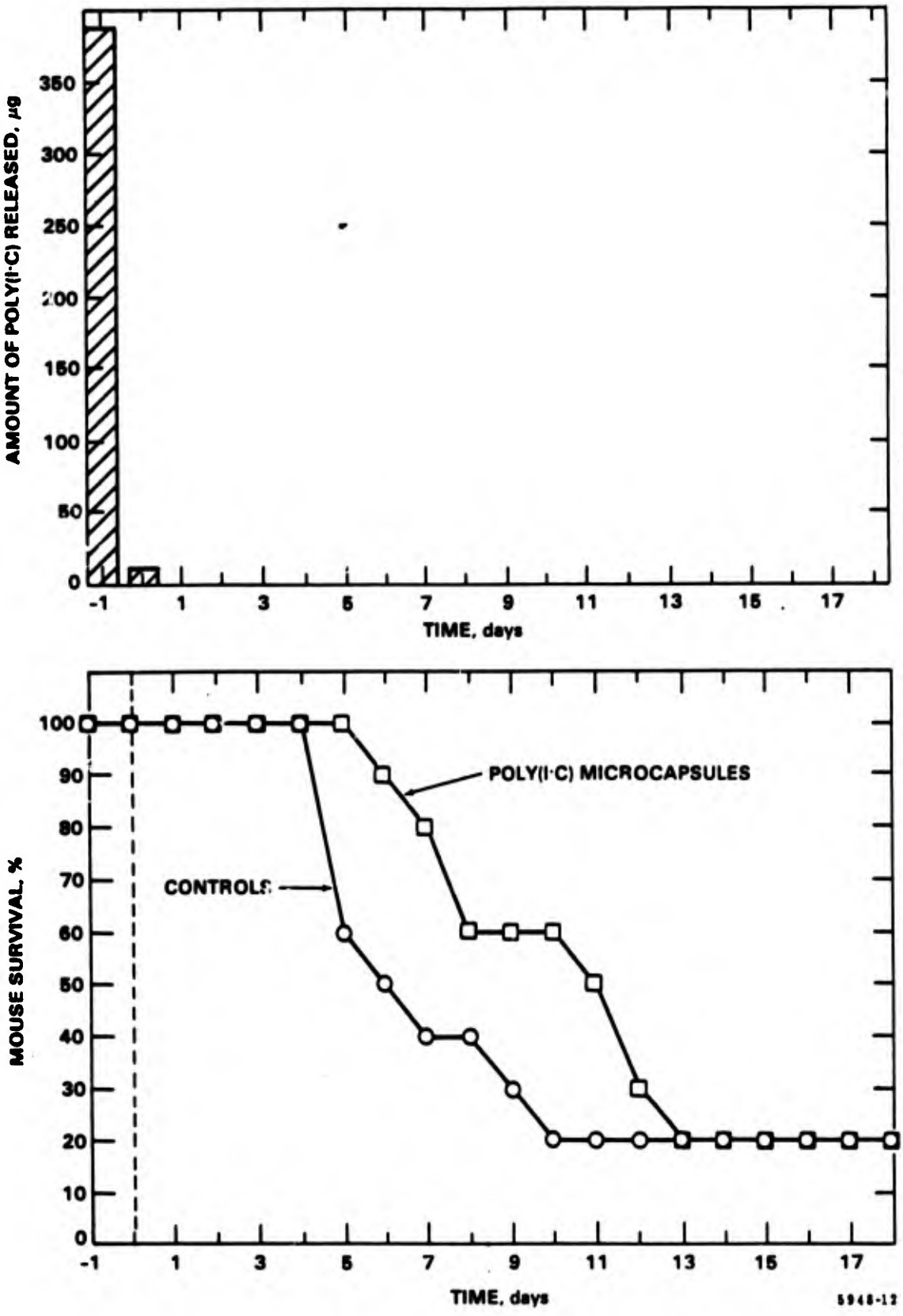


Figure 7. Daily in vitro release kinetics (above) and survival rate (below) for mice infected with Rift Valley Fever virus and treated with 500 µg of encapsulated polv(I·C): Batch D763-013.

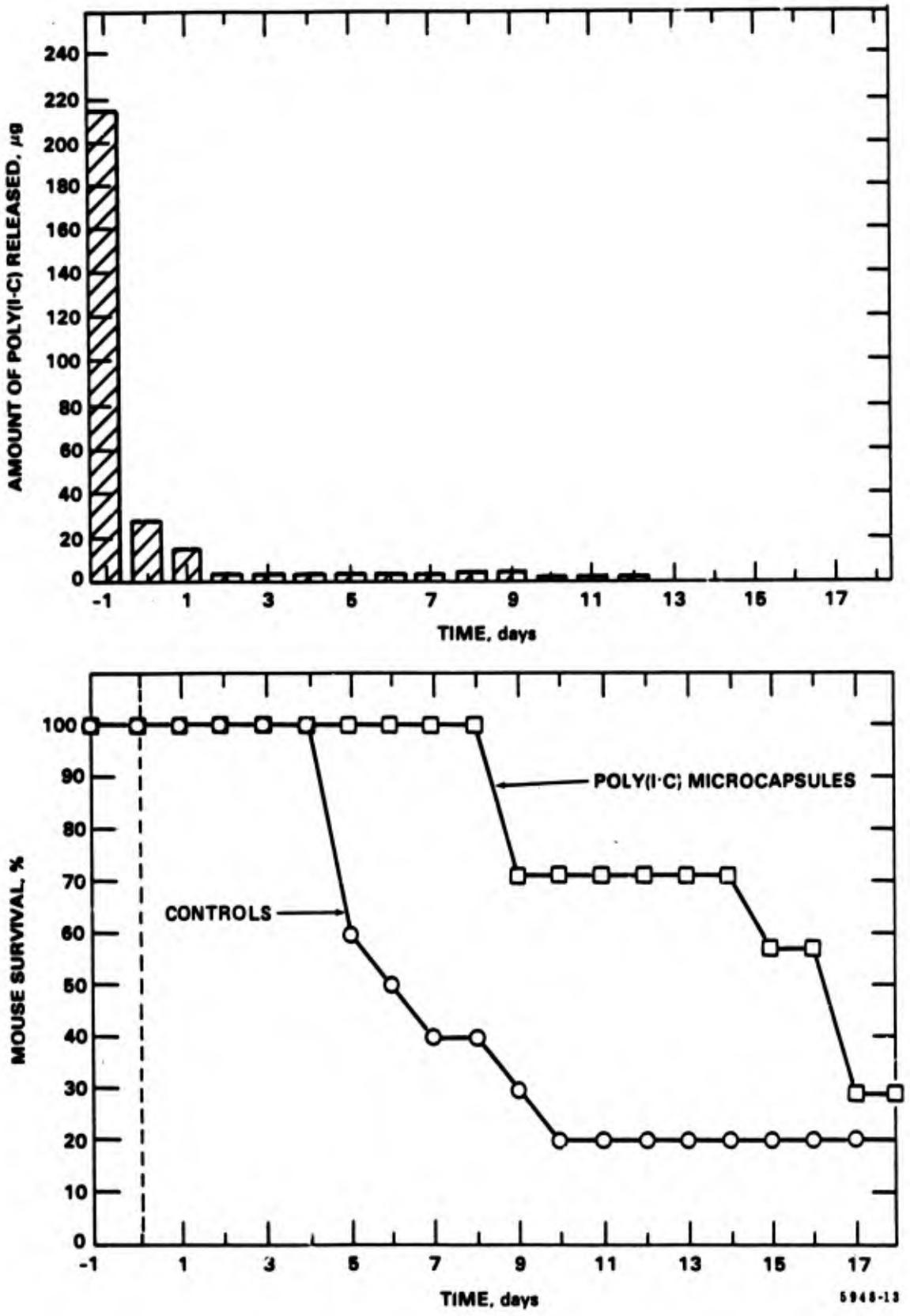


Figure 8. Daily in vitro release kinetics (above) and survival rate (below) for mice infected with Rift Valley Fever virus and treated with 500 µg of encapsulated poly(I·C): Batch D763-117.

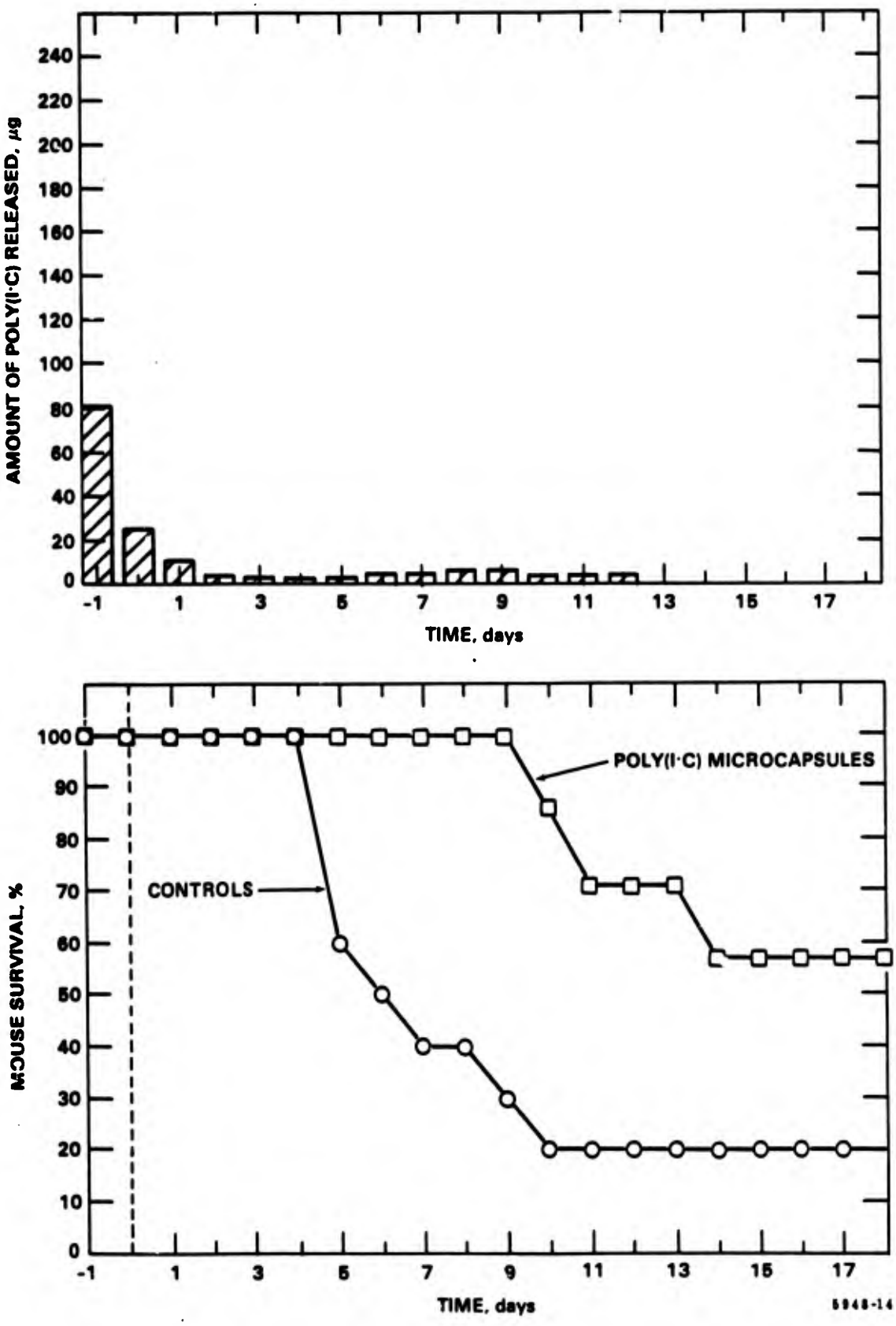


Figure 9. Daily in vitro release kinetics (above) and survival rate (below) for mice infected with Rift Valley Fever virus and treated with 500 µg of encapsulated poly(I·C): Batch D763-121.

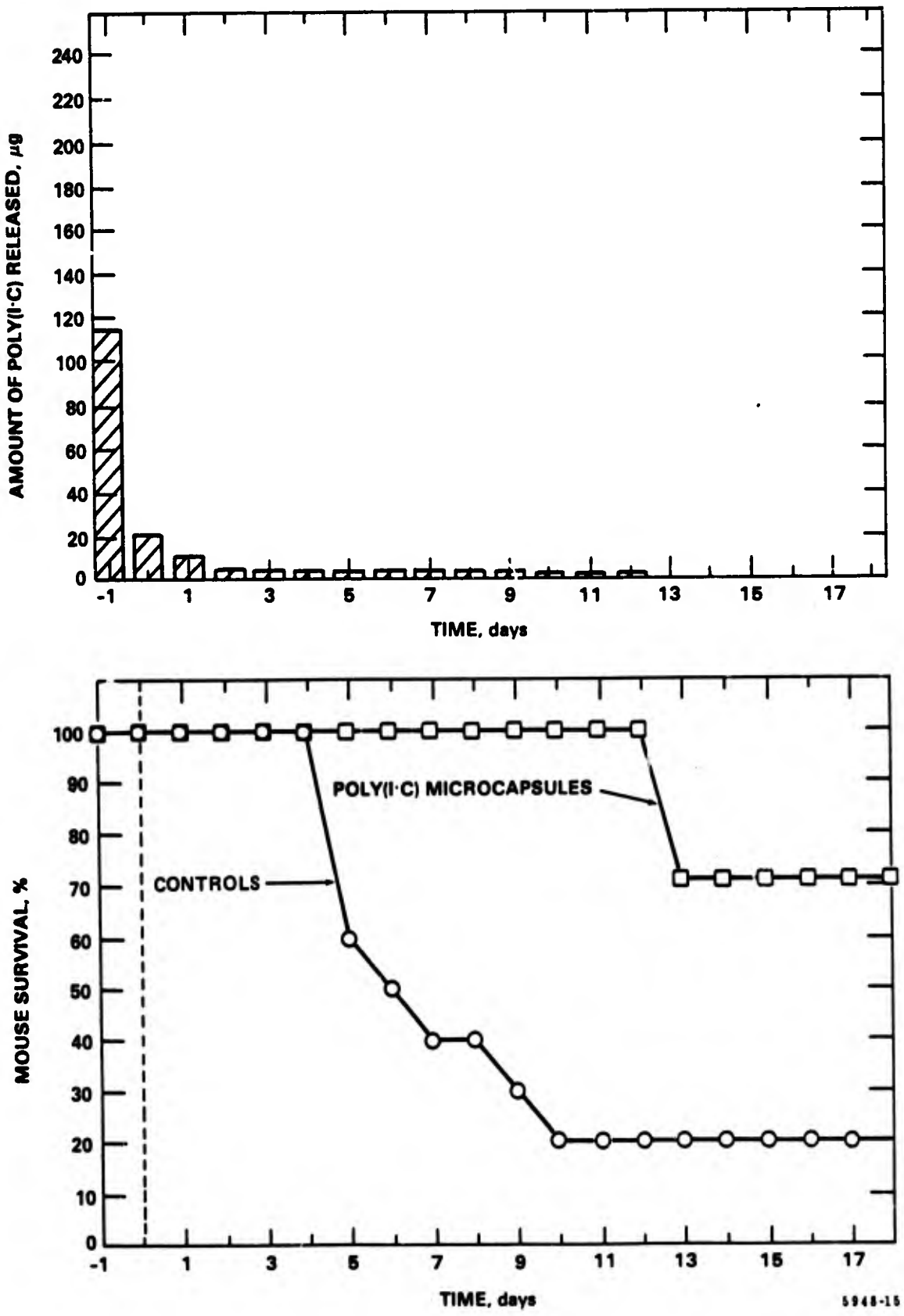


Figure 10. Daily in vitro release kinetics (above) and survival rate (below) for mice infected with Rift Valley Fever virus and treated with 500 µg of encapsulated poly(I·C): Batch D763-125.

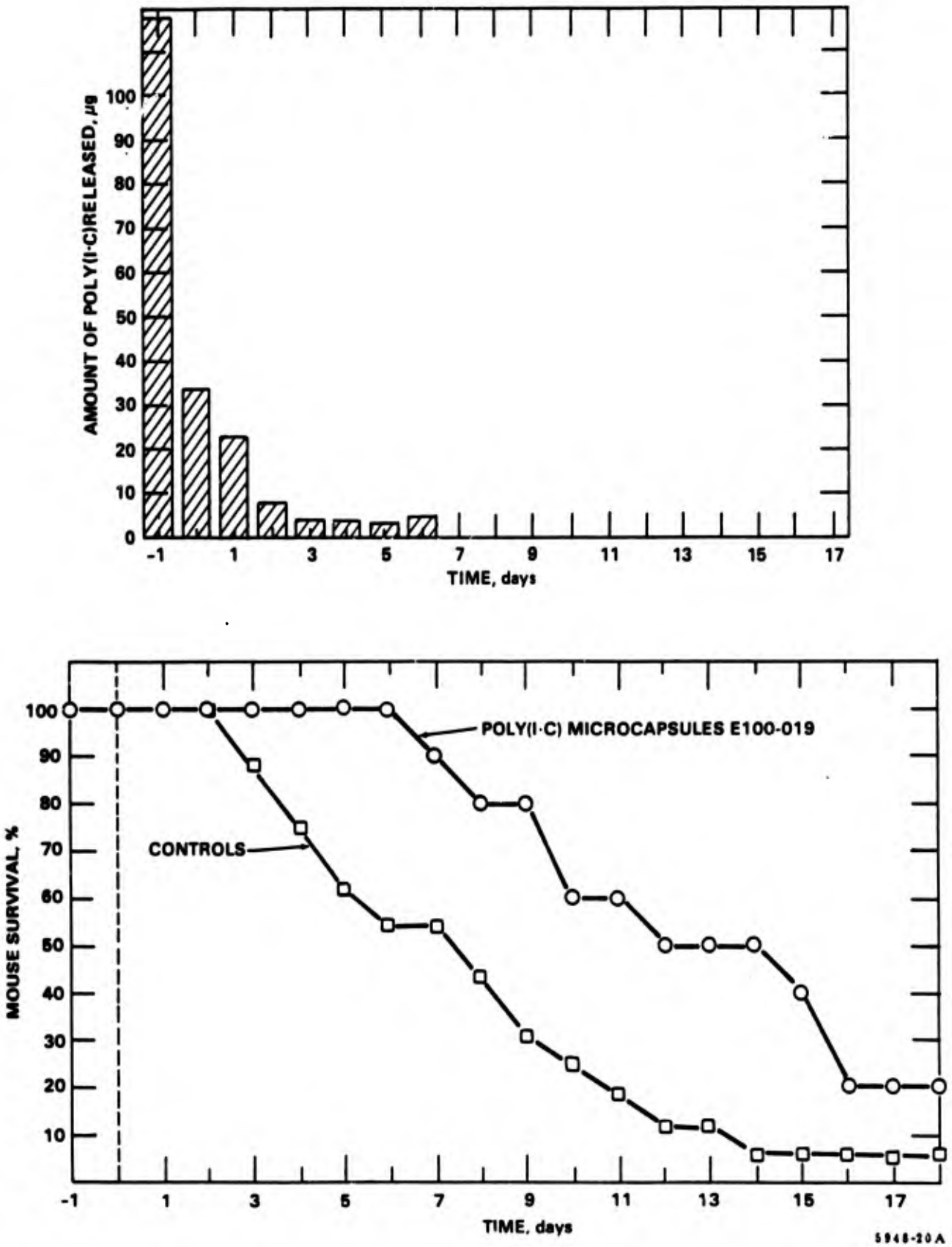


Figure 11. Daily in vitro release kinetics (above) and survival rate (below) for mice infected with Rift Valley Fever virus and treated with 200 µg of encapsulated poly(I·C): Batch E100-019.

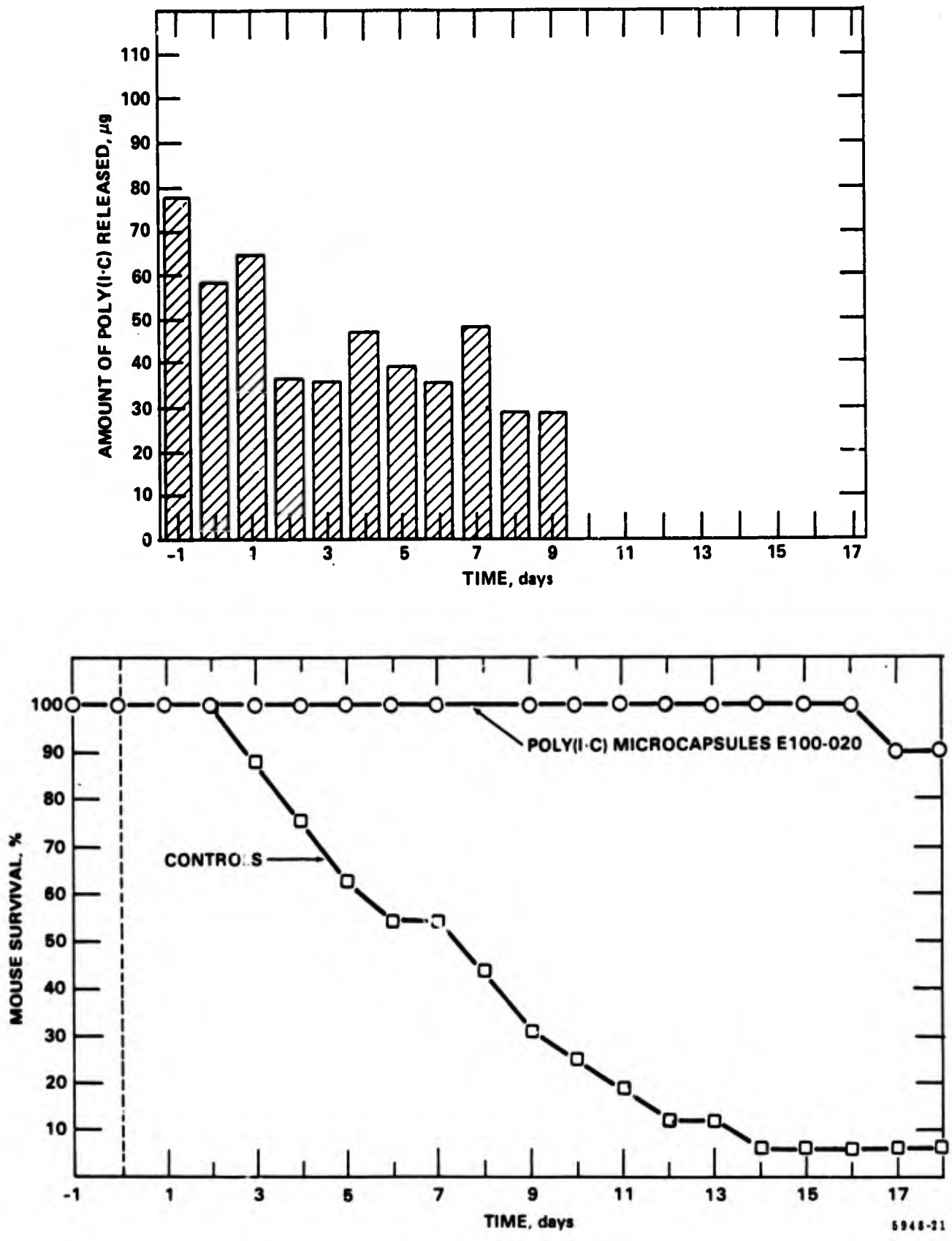


Figure 12. Daily in vitro release kinetics (above) and survival rate (below) for mice infected with Rift Valley Fever virus and treated with 500 µg of encapsulated poly(I·C): Batch E100-020.

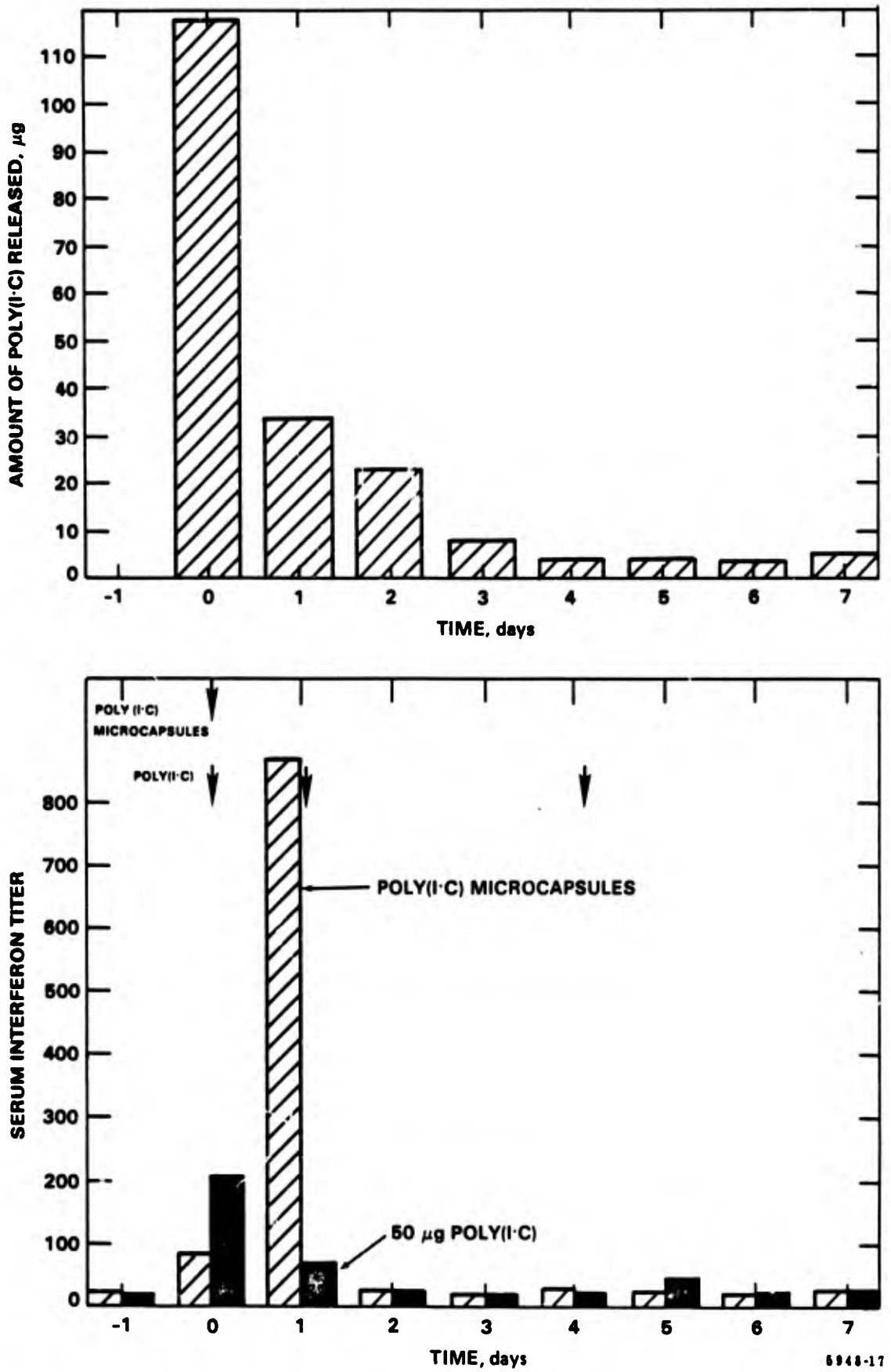


Figure 13. Daily in vitro release kinetics (above) and interferon levels (below) for mice treated with 200 µg of encapsulated poly(I·C): Batch E100-019.

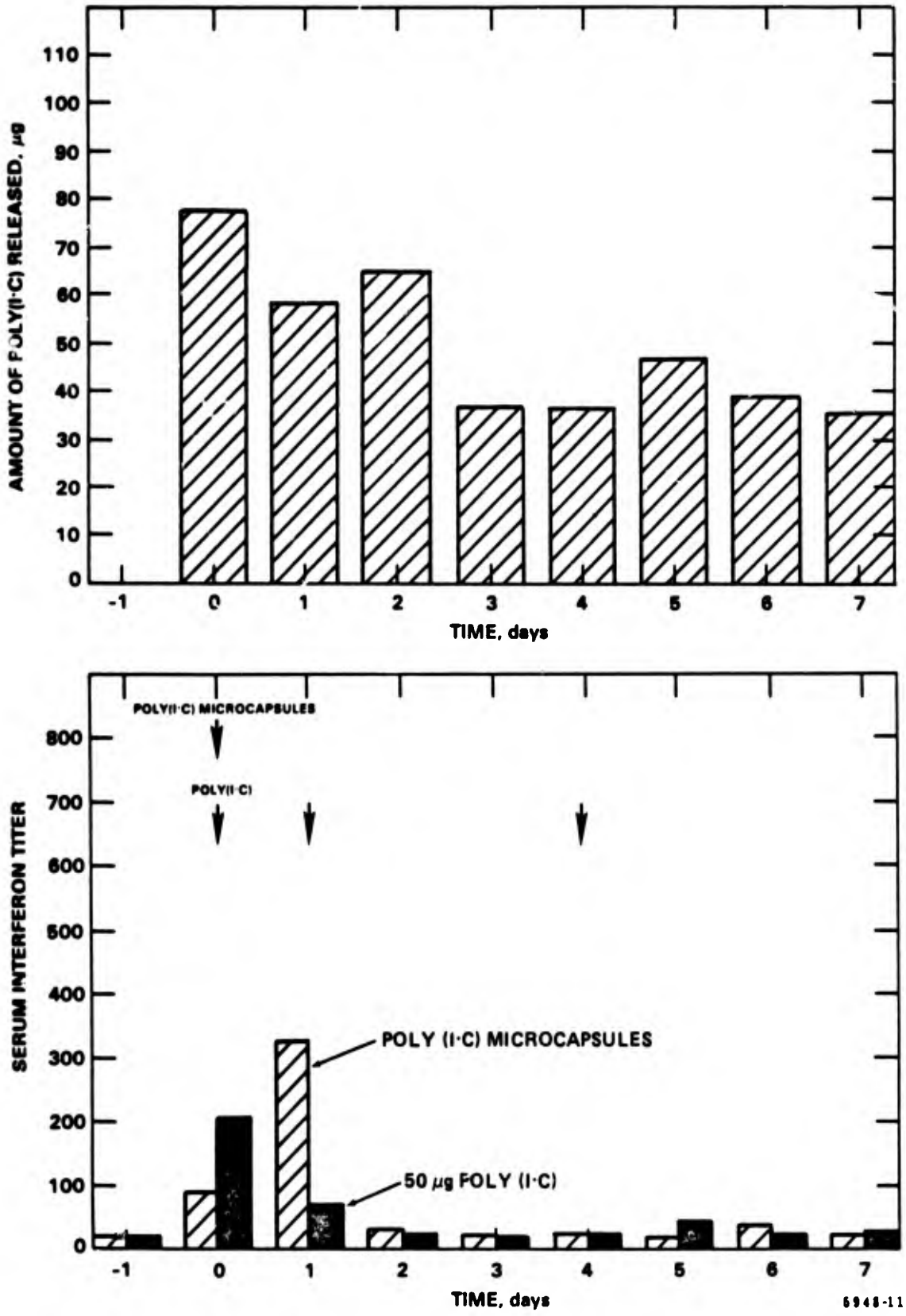
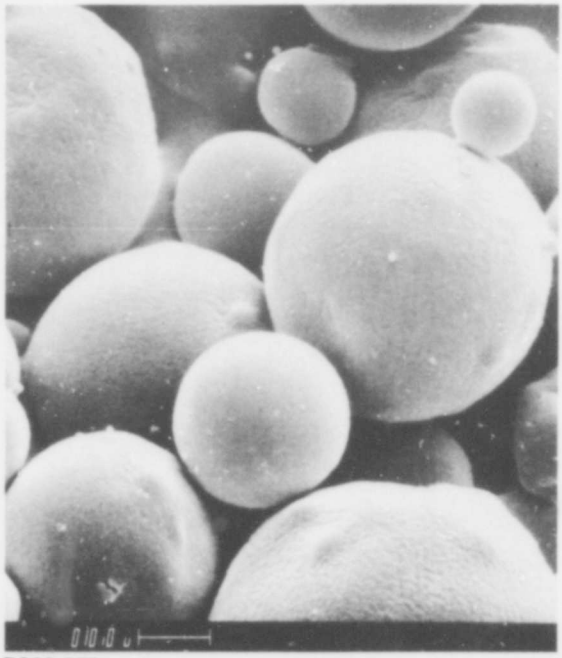
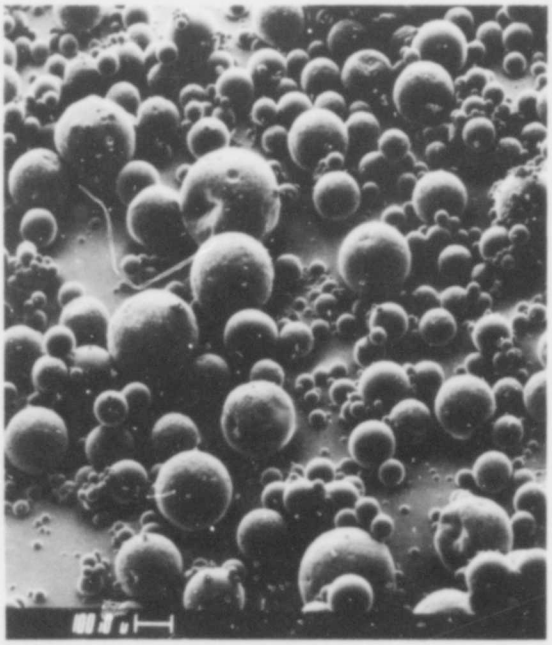


Figure 14. Daily in vitro release kinetics (above) and interferon levels (below) for mice treated with 500 µg of encapsulated poly(I·C): Batch E100-020.

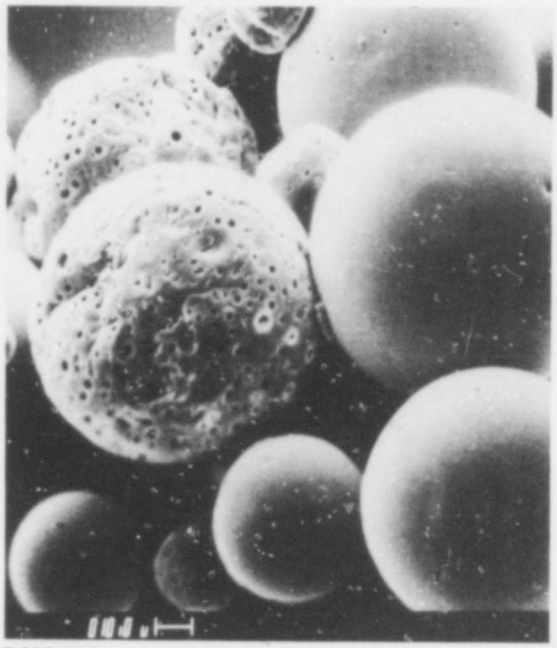
D902-078 50X



D902-078 1000X



D902-119 50X



D902-119 500X

5948-18

Figure 15. Photomicrographs taken by scanning electron microscopy of prototype JE vaccine microcapsules prepared with a DL-PLG excipient: Batches D902-078 and D902-119.

DISTRIBUTION LIST

5 copies	Commander US Army Medical Research Institute of Infectious Diseases ATTN: SGRD-UIZ-M Fort Detrick, Frederick, MD 21701-5011
1 copy	Commander US Army Medical Research and Development Command ATTN: SGRD-RMI-S Fort Detrick, Frederick, MD 21701-5012
2 copies	Defense Technical Information Center (DTIC) ATTN: DTIC-DDAC Cameron Station Alexandria, VA 22304-6145
1 copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20814-4799
1 copy	Commandant Academy of Health Sciences, US Army ATTN: AHS-CDM Fort Sam Houston, TX 78234-6100