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Immunization with a vaccine against Venesuelan equine encephalitis (VEE) virus encapsulated in microspheres formulated of the biodegradable and biocompatible copolymer poly(DL-lactide-co-glycolide) (DL-PLG) was investigated. Whole ^{60}Co-inactivated and formalin-fixed TC 83 VEE virus was encapsulated in DL-PLG of equal mole parts lactide and glycolide (50:50 DL-PLG) by an emulsion-based process to yield microspheres of approximately 1 to 10 μm in diameter and containing approximately 0.8% by weight VEE vaccine. As measured by ELISA, immunization of mice with from 3 to 100 μg of microencapsulated vaccine potentiated the plasma IgG anti-VEE antibody response relative to the same dose of vaccine administered in PBS. Potentiation of the response was most pronounced when the amount of vaccine was limiting, but was evident at all doses tested. However, plasma IgM and IgA anti-VEE antibody levels were not significantly enhanced by vaccine administration in microspheres. *In vitro* neutralization of virus infectivity assays confirmed that the enhanced antibody responses stimulated by vaccine delivery in the 50:50 DL-PLG microspheres resulted in increased virus neutralization activity.

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INTRODUCTION

Numerous infectious agents are acquired in nature through aerosol exposure. Included in this group are several of the 97 arboviruses and roboviruses which are pathogenic for humans. Although vaccines have been produced and systematically tested for only a few of these viruses, there is evidence that the form of the vaccine determines the extent to which protection against aerosol exposure is provided. A case in point are the Venezuelan equine encephalitis virus (VEE) vaccines TC-83 and C-84. Immunization of hamsters with the live attenuated strain TC-83 confers protection against both aerosol and subcutaneous challenge with virulent VEE, while multiple immunizations with the formaldehyde-killed form, C-84, is only protective when challenge is performed by the subcutaneous route (1). Thus, the inactivated vaccine for this virus suffers from both poor immunogenicity and an inability to induce protection against viral penetration through the respiratory tract, even when circulating antibodies are induced.

The successful protection against pulmonary exposure afforded by the live-attenuated VEE strain demonstrates that vaccines of this type can be extremely effective. However, the production and testing of live virus vaccines is exceedingly time consuming and administratively difficult. An approach which could overcome the deficiencies in the immunogenicity of inactivated whole virus vaccines, particularly with respect to antibodies in the respiratory tract, would thus make the development of vaccines to a wide range of airborne viral pathogens substantially easier to accomplish.

Antibodies which are present within the respiratory tract originate from two different sources. The bronchioli and alveoli predominantly contain IgG which is passively derived from the intravascular pool (2,3). In contrast, secretory IgA (sIgA) predominates in the mucus which bathes the nasopharynx and bronchial tree (4-6). The bulk of bronchial IgA is locally produced (2) by IgA plasma cells which line the lamina propria of the airway wall and are particularly concentrated about the bronchial glands (4). Importantly, numerous investigations have shown that sIgA levels in humans (7,8) and mice (9,10) correlate with protection against pulmonary viral challenge to a significantly greater extent than do circulating antibody titers.

Unlike the blood circulation, the induction of antibodies at the mucosa of the upper respiratory tract is not commonly achieved through parenteral immunization with inactivated vaccines. Secretory IgA antibodies are, in general, only induced through direct immunization of mucosally-associated lymphoid tissues (MALT), such as those found in the gastrointestinal tract and bronchial tree (11-13). One possible reason for the inability of the C-84 VEE virus vaccine to protect against aerosol exposure may be the failure of this parenterally-administered vaccine to induce sIgA antibodies. However, simple mucosal application of C-84 is not an effective strategy because of antigenic degradation at these surfaces, and inefficient adsorption into the MALT. Thus, immunization through MALT with an inactivated vaccine to protect against the aerosol spread of a virus such as VEE requires a method of vaccine delivery which will protect the antigen from degradation in the gut or respiratory tract lumen, enhance its uptake into the MALT and potentiate the subsequent immune response. One vaccine delivery method which has the potential to provide these characteristics is microencapsulation.

Microencapsulation involves the coating of a bioactive agent, such as a vaccine, in a protective wall material which is generally polymeric in nature. The microsphere product is a free-flowing powder of spherical particles which can be produced across a size range from $\leq$1 μm to as large as 3 mm in diameter. The particular system investigated in the studies reported here involves the use of poly(DL-lactide-co-glycolide) (DL-PLG) copolymers (14-

16). DL-PLG is in the class of copolymers from which resorbable suturs, resorbable surgical clips and controlled-release drug microspheres and implants are made (17). These biocompatible polyesters are approved for and have a 30 year history of safe use in humans. After introduction into the body, DL-PLG induces only a mild inflammatory response and biodegrades through hydrolysis of ester linkages to yield the normal body constituents lactic and glycolic acids (18, 19). Further, the rate at which DL-PLG biodegrades is a function of the ratio of lactide to glycolide in the copolymer (20), thus determining the time after administration when vaccine release initiates, and the subsequent rate of release (16).

We, and others, have shown that the systemic injection of staphylococcal enterotoxin B toxoid (14-16, 21-23), influenza vaccine (24), simian immunodeficiency virus (SIV) vaccine (22) or ovalbumin (25, 26) encapsulated in 1-10 μm DL-PLG microspheres results in a strongly potentiated antibody response. In the case of SEB toxoid, mice immunized with 50 μg of vaccine in microspheres mounted a neutralizing plasma anti-toxin response which was equivalent in level and duration to that induced by the same dose of toxoid in complete Freund's adjuvant (CFA), but without an inflammatory response (14). Similar immunopotentiation has been obtained in rhesus macaques immunized by the intramuscular injection of microencapsulated SEB toxoid or whole formalin-inactivated SIV (22). Further, mixtures of microspheres with varying sizes and/or lactide to glycolide ratios have been used to deliver multiple discrete releases of vaccine following a single injection (16). Thus, this system the formulation of a vaccine into an adjuvant which will deliver primary and timed booster immunizations in a single administration.

DL-PLG microspheres have also been found to be an effective vehicle for mucosal immunization via the oral (15, 16, 21, 23) and intratracheal (22, 23) routes. This activity is attributable to the protection against nonspecific and specific proteolytic degradation provided by the encapsulation, as well as the enhanced and targeted delivery of the intact vaccine into the MALT. This adsorption of microspheres into the MALT from the lumen of the gut or respiratory tract is through a phagocytic-like mechanism restricted to particles of $\leq$10 μm in diameter (15, 16, 21).

In this report results are presented which relate to the development of a microencapsulated VEE vaccine based on inactivated whole virus. The objectives of the studies under this contract effort are to investigate the potential advantages of: 1) a single injection multiple release microsphere adjuvant formulation for VEE vaccine, and 2) mucosal immunization with microencapsulated VEE vaccine. Included in the later are investigations of oral, intratracheal and intranasal immunization both singly and as boosters following systemic priming. To date, process conditions have been determined which allow the microencapsulation of whole ^{60}Co-inactivated VEE in 1 to 10 μm microspheres formulated with 50:50 DL-PLG, characteristics which have been shown with other antigens to provide maximal potentiation of the antibody response and adsorption into MALT (14, 16). Since the encapsulation process includes a step which requires the use of an organic solvent, process conditions have been developed for two solvents, methylene chloride and ethyl acetate. *In vivo* immunogenicity testing has shown that VEE vaccine microspheres produced with either process solvent provide a 4 to 32-fold potentiation of the IgG anti-VEE response as determined by ELISA and that microspheres produced with ethyl acetate may have greater activity . Further, neutralization of viral infectivity assays suggest that the microspheres produced with ethyl acetate may be significantly better at the induction of neutralizing antibodies. These results are discussed in relation to the physical characteristics of the immunizing VEE vaccine microsphere batches.

MATERIALS AND METHODS

Mice. Specific pathogen free BALB/c mice of mixed sexes were used throughout these experiments. They were bred and maintained in our barrier facilities at the University of Alabama at Birmingham. They were allowed food and water *ad libitum*, and were entered into experimental protocols at 8-12 weeks of age.

VEE virus vaccine. The VEE virus vaccine used in these studies was the attenuated vaccine strain TC-83 which was grown in BHK-21 cells in the laboratories of Dr. Jonathan F. Smith in the Department of Viral Biology, USAMRIID, Ft. Detrick, MD. After a single passage to expand the infectious stock of virus, monolayers of cells in 850 cm^2 roller bottles were infected at an MOI of 1 and cultured for 24 hr. The culture supernatants were harvested and clarified by centrifugation (10,000 x g) for 20 min at 4°C prior to bringing the solution to a concentration of 7.0 % polyethylene glycol and 0.5 M NaCl. Virus was allowed to precipitate from this solution overnight at 4°C, after which the precipitate was packed by centrifugation . Following resuspension in a minimal volume of H_2O, the virus was isolated by banding in a 20 to 60 wt/wt % sucrose gradient. Fractions containing virus free of contaminants were selected on the basis of SDS polyacrylamide electrophoresis gels stained with coomasie brilliant blue. The pure virus was fixed by bringing the suspension to 1:4,000 in reagent grade formaldehyde (37 %) for 24 hr at room temp, washed and frozen as a wet pellet which was inactivated by ^{60}Co irradiation.

Microencapsulation of VEE Virus Vaccine and Characterization of the Microspheres. The formalin-fixed and ^{60}Co inactivated pellet of VEE virus vaccine was thawed, suspended in PBS to a concentration of approximately 30 mg/ml protein and the exact protein concentration determined using the BCA assay (Pierce Chemical Company, Rockford, IL). The vaccine was microencapsulated by an emulsion-based process (14) in which the excipient solvent, either methylene chloride or ethyl acetate, was removed by extraction. The surface morphology of each batch of microspheres was examined from photomicrographs obtained by scanning electron microscopy. This confirmed that a smooth surface of continuous polymeric coating had been obtained in each case. The vaccine content (core loading) was determined by dissolving a sample of the microspheres in 1.0 M sodium hydroxide determining the amount of antigen obtained and calculating the percent antigen by weight. Size distributions of each batch of microspheres were determined using a particle size analyzer (Malvern Instruments, Malvern, United Kingdom). The results of these analyses were plotted to show the number fraction of the microspheres in each lot having given diameters and were calculated and plotted to show the number fraction having a given volume. *In vitro* vaccine release kinetics were determined by placing a sample of the microspheres in a receiving fluid consisting of 0.5 M phosphate (pH 6.8), and the buffer was exchanged at 6 hr, 24 hr and every 24 hr thereafter until termination of the study. The amount of protein in the receiving fluids was quantified and related to the total protein in the sample of microspheres to determine the cumulative percent antigen release as a function of time.

Immunizations and Collection of Plasma Samples. Microencapsulated and nonmicroencapsulated VEE virus vaccine was suspended in PBS for administration. Mice were immunized by subcutaneous (SC) injection of the adjuvanted or free antigen in a total volume of 0.25 ml. At the times indicated in the text and figures, blood was collected in calibrated heparinized capillary pipettes by retroorbital puncture under anesthesia, and the plasma harvested following centrifugation. All samples were frozen until assayed for antibody activity.

Enzyme-linked Immunosorbent Assays of Plasma IgM, IgG and IgA Anti-VEE Virus Antibodies. Enzyme-linked immunosorbent assays (ELISAs) of VEE virus-specific IgM, IgG and IgA antibodies in plasma samples were carried out in rigid 96 well assay plates (Pro-Bind, Becton Dickinson, Lincoln Park, NJ) coated overnight with freeze fractured VEE virus at 1 μg/ml in borate-buffered saline (BBS). All washing steps employed PBS containing 0.05% Tween 20 (PBS-Tween) and the diluent for all samples and reagents was PBS-Tween with 1% BSA. After blocking, serial 2-fold dilutions of the plasma samples, in triplicate, were added and incubated at 25°C for 6 hr. The VEE virus-binding antibodies were detected by sequential incubation with optimal dilutions of biotinylated goat anti-mouse IgM, IgG or IgA heavy chain-specific antibody overnight at 4°C (Southern Biotechnology Associates, Birmingham, AL), horseradish peroxidase-streptavidin for 2 hr at 25°C and the substrate 2,2'-azino-di-(3-ethyl-benzthiazoline-sulfonic acid) at 0.3 mg/ml in pH 4.0 citrate buffer containing 0.0003% H_2O_2. The developed color was read after 15 min at 405 nm on a model EL312 kinetics reader (Bio-Tech Instruments, Inc., Winooski, VT) and the results presented as the reciprocal of the greatest sample dilution producing a signal significantly greater than that of the group-matched prebleed at the same dilution (endpoint titration).

Neutralization of VEE Virus Infectivity. Selected plasma samples from mice immunized with microencapsulated and nonmicroencapsulated VEE virus vaccine were tested for neutralization of VEE virus infectivity. These *in vitro* assays were performed in the laboratories of Dr. J. F. Smith, USAMRIID, Ft. Detrick, MD.

RESULTS

Microencapsulation of VEE Virus Vaccine. Previous experiments with microencapsulated vaccines for SEB (14, 16), SIV (22) and influenza virus (unpublished observations) demonstrated that maximal potentiation of antibody responses was obtained when the DL-PLG microspheres were of a 1 to 10 μm size range and when the vaccine was released through hydrolysis of the DL-PLG rather than by a process of rapid diffusional release. In addition, responses to the viral vaccines were enhanced to the greatest extent when formalin treatment was employed in their preparation, appearantly because this fixation stabilized labile epitopes in the envelope glycoproteins during the encapsulation process. Further, microencapsulation process solvents were observed to effect the immunogenicity of different vaccines to different extrents. Therefore, formalin-fixed VEE virus vaccine was encapsulated using methylene chloride and ethyl acetate-based processes. The process conditions were in each case manipulated in an effort to yield vaccine-containing microspheres with optimal physical characteristics.

Three batches of microspheres were selected for *in vivo* evaluation based on their size distribution and release characteristics (Table 1). Batches G320-005-00 (Fig. 1) and G320-018-00 (Fig. 2) were produced using methylene chloride and were similar in that they retained 90% or more of the vaccine as meassured in the *in vitro* antigen release assay and were of a size range in which 90% of the internal volume was within microspheres of less than 17 μm in diameter. Batch G320-007-00 (Fig. 3) was produced using ethyl acetate and retained 86% of the vaccine, but was of a larger than ideal size distribution.

DL-PLG Microspheres Potentiate the Antibody Response to VEE Virus Vaccine. Groups containing 6 BALB/c mice were subcutaneously (SC) injected with 100, 50, 25 or 12.5 μg of VEE virus vaccine encapsulated in microsphere batch G320-005-00 (methylene chloride process solvent; 90 vol % <17.7 μm). For comparison, groups of mice were immunized with identical doses of non-microencapsulated vaccine. The mice were bled prior to immunization and at 10 day intervals through experimental day 100. On day 50 the mice were boosted by the SC injection of the same dose and form of VEE vaccine used for their primary immunization. All plasma samples were tested in isotype-specific ELISAs for their titers of IgM, IgG and IgA antibodies which bound solid-phase adsorbed freeze-fractured VEE virus.

The levels of IgM and IgA antibodies were found to be low and unremarkable regardless of the form or dose of vaccine administered (data not shown). However, substantial IgG anti-VEE virus responses were detected in all of the immunization groups (Fig. 4). The non-microencapsulated vaccine stimulated primary IgG anti-VEE virus antibody levels which were equivalent across the range of immunizing doses examined, with a maximal titer of 102,400 generally being attained 20 to 40 days post immunization. In contrast, primary immunization with the microencapsulated VEE vaccine stimulated peak plasma IgG antibody levels which were 4 to 32-fold higher than those attained by the free vaccine at equivalent doses. Following secondary immunization the fold increase in the antibody responses provided by the microencapsulated vaccine was not as dramatic as that observed after primary immunization. However, the generally observed 2 to 4-fold increase in the levels of secondary antibodies represents a further potentiation in the response when absolute antibody levels are considered.

A second preparation of microspheres prepared with methylene chloride (Batch G320-018-00; 90 vol % <15.8 μm) was evaluated across a lower range of vaccine doses. Groups of

mice were immunized by SC injection with 25, 12.5, 6.25 or 3.12 µg doses of microencapsulated and non-microencapsulated vaccine on day 0, and boosted with the same dose and form of vaccine on day 50. As was observed with higher vaccine doses, the plasma IgM and IgA responses were quite low. However, substantial IgG levels were obtained following immunization with even the 3.12 µg doses (Fig. 5). At all antigen doses tested the microencapsulated form of the vaccine was found to provide significant enhancement of both the primary and secondary anti-VEE virus responses. These increased antibody levels were most pronounced at very limiting antigen doses, and late in the secondary response. In fact, across the 3.12 to 100 µg range of microencapsulated vaccine doses tested no indication of a fall in the secondary IgG anti-VEE virus titers was ever detected through the 100 days of the experiments.

Comparison VEE Virus Vaccine Immunogenicity in Microspheres Processed with Methylene Chloride or Ethyl Acetate. The immunogenicity of VEE virus vaccine in microspheres produced by processes which employed methylene chloride or ethyl acetate as the solvent for the DL-PLG were compared. Groups of mice were immunized by the SC injection of 25 µg of vaccine in 50:50 DL-PLG microspheres from Batches G320-018-00 (methylene chloride process solvent; 90 vol % <15.8 µm) or G320-007-00 (ethyl acetate process solvent; 90 vol % <48.6 µm) on day 0 and boosted with the same microsphere batch and vaccine dose on day 50. Quantitation of the plasma IgG anti-VEE virus antibody response demonstrated that the microspheres produced with ethyl acetate stimulated both primary and secondary responses which consistently exceeded those seen after immunization with the vaccine encapsulated by the process using methylene chloride (Fig. 6). Although this difference never exceeded the degree of error inherent in the end-point titration assay, it appears clear from a comparison of the titration curves and the consistency of the difference over time that the greater degree of immune enhancement provided by the ethyl acetate processed microspheres is real. In addition, the size distrabution of the microspheres produced using ethyl acetate is larger than the ideal 1 to 10 µm size range identified in other experimental systems. Thus, the advantage provided by encapsulation using ethyl acetate may be greater than is indicated by this experiment.

DL-PLG Microspheres Potentiate the VEE Virus Neutralizing Response . The ability of immune sera to neutralize the infectivity of virus is a measure of immunity which often provides a higher correlation with protection from challenge than do total anti-virus antibody levels. Assays to quantitate the neutralization of viral infectivity activity in the plasma samples from selected immunization groups were performed in the laboratories of Dr. J. F. Smith, USAMRIID, Fort Detrick, MD. In these assays the neutralizing activity of plasma samples from mice immunized with 25 µg of nonmicroencapsulated VEE vaccine and that in the plasma of mice immunized with 25 µg of vaccine in each of the three prototype microsphere formulations were compared to the neutralizing activity in the plasma of mice which had been immunized with 100 µg of vaccine emulsified in complete Freund's adjuvant. All immunizations were by SC injection and all groups were boosted on experimental day 50 with the same dose and form of vaccine used for primary immunization.

Mice receiving 100 µg of VEE virus vaccine as an emulsion in CFA produced a neutralizing titer of 640 after the primary immunization and of 1,280 after secondary immunization (Fig. 7). This immunization group provides a known strongly positive standard against which to judge the alternate immunization procedures. By comparison,

those mice which received 25 µg of vaccine in PBS mounted a much weaker response and did not exhibit neutralizing antibodies until 20 days after secondary immunization. The maximum titer of 160 induced by the free vaccine was attained on experimental day 70 and thereafter it fell to a titer of 40 on day 100. Mice immunized with 25 µg of vaccine in either of the batches of DL-PLG microspheres manufactured using methylene chloride as the process solvent did not attain a neutralizing response as high as that seen following immunization with the vaccine in PBS. Microsphere Batch G320-018-00 did not stimulate a detectable neutralizing response at any time tested. Batch G320-005-00 exhibited more activity, but only induced the appearance of neutralizing antibodies at a titer of 40 after administration of the booster dose. In contrast, microsphere Batch G320-007-00, which was produced using ethyl acetate as the process solvent, demonstrated a substantially enhanced ability to stimulate neutralizing antibodies relative to the vaccine in PBS (Fig. 7). Neutralizing activity was detected 20 days after primary immunization and this activity continued to rise through day 40. Secondary immunization resulted in a prompt boosting to a titer of 320 on experimental days 70, 80 and 90. Thus, ethyl acetate appears to be a better solvent for the production of VEE virus vaccine microspheres as determined by both total and neutralizing anti-virus titers.

DISCUSSION AND CONCLUSIONS

Available evidence indicates that protective immunization against infection by airborne VEE virus will require that an inactivated vaccine be incorporated into an improved adjuvant and/or delivery system. The areas in which this vaccination could be most readily improved are an enhancement in the level of the circulating anti-viral antibody response and the induction of sIgA antibodies in the secretions which bathe the upper respiratory tract. Results obtained in the first half of this contract effort indicate that it will be possible to address the deficiencies in inactivated VEE vaccines through the use of a microsphere delivery system.

Process conditions have been developed which allow the encapsulation of ^{60}Co-inactivated and formalin-fixed whole VEE virus vaccine in microspheres formulated with the biodegradable and biocompatible copolymer DL-PLG. These conditions have been derived for two process solvents, methylene chloride and ethyl acetate, each of which has proven to be appropriate for the encapsulation of at least one other inactivated whole virus-based vaccine. These prototype vaccine microspheres contain between 0.5 and 1.0 % vaccine by weight, show a uniform surface morphology and minimal vaccine release through imperfections in the microsphere polymer wall, and approach the ideal size distribution of 1 to 10 μm in diameter. Thus, using two different process solvents the VEE virus vaccine has been encapsulated in microspheres with physical characteristics which have been characterized as providing maximal potentiation of the humoral response and adsorption into MALT.

In vivo evaluation of the immunogenicity of the prototype microspheres following SC injection confirmed that significant enhancement of the circulating anti-VEE virus antibody response was provided by all three microsphere preparations. These ELISA-based measurements demonstrated that the vast majority of this response was of the IgG class, that microspheres enhanced this response across a wide range of vaccine doses and suggested that processing with ethyl acetate may provide microspheres with greater immune enhancing activity. This later observation was confirmed and extended by assay of the neutralization of VEE virus infectivity. Plasma samples from mice SC immunized with 25 μg of microencapsulated vaccine clearly showed the microspheres processed with ethyl acetate to be more effective. The ethyl acetate processed microspheres induced a neutralizing response after primary immunization and the level of neutralizing activity after secondary immunization approached that of mice receiving two doses of vaccine in CFA.

Experiments are currently in progress which compare the immunogenicity of four microsphere preparations at several vaccine doses. These preparations test the four combinations of two variables, methylene chloride versus ethyl acetate as the process solvent and fixation versus no fixation of the VEE virus vaccine. The first variable is being retested to confirm the previously observed enhanced immunogenicity of the vaccine in microspheres prepared with ethyl acetate, and the second variable is being tested because of the reported destruction of at least one VEE virus neutralizing epitope by formalin treatment. As before, both total and neutralizing anti-VEE virus antibodies will be measured. In addition, plans have been made for Dr. Smith's group to perform infectious challenges of these mice. It is anticipated that this experiment will provide the data required for selection of an optimal solvent and vaccine combination with which to proceed. At the present time there do not appear to be any impediments to the testing of mucosal immunization with microencapsulated VEE virus vaccine or the preparation of a multi-release microsphere formulation for systemic administration.

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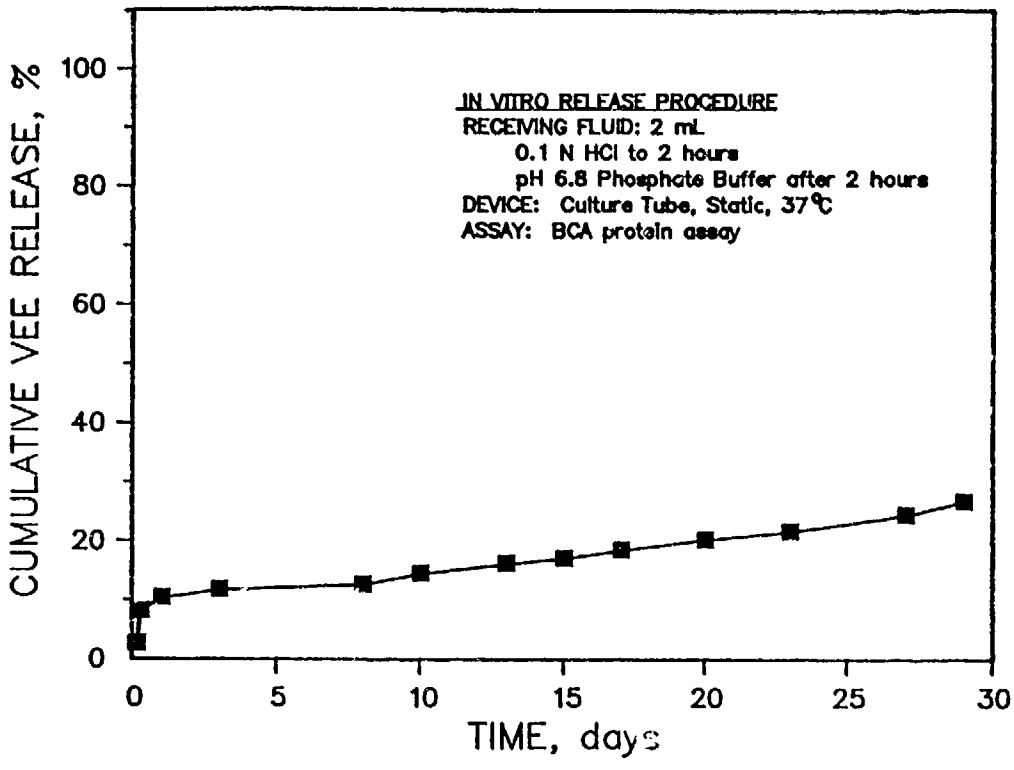
TABLE 1. VEE Virus-Containing Microspheres Selected for In Vivo Evaluation

Batch	VEE virus treatment	Excipient solvent	Antigen loading wt % protein	In vitro release (% at 24 hr)	Size	
					% ≤ 10 μm	90 vol %[a]
G320-005-00	Formalin fixed + ^{60}Co irradiated	Methylene chloride	0.81	10.4	45.9	<17.67 μm
G320-018-00	Formalin fixed + ^{60}Co irradiated	Methylene chloride	0.78	3.0	69.8	<15.8 μm
G320-007-00	Formalin fixed + ^{60}Co irradiated	Ethyl acetate	0.78	13.8	42.5	<48.6 μm

[a]90% of the internal volume contained within microspheres of diameter.



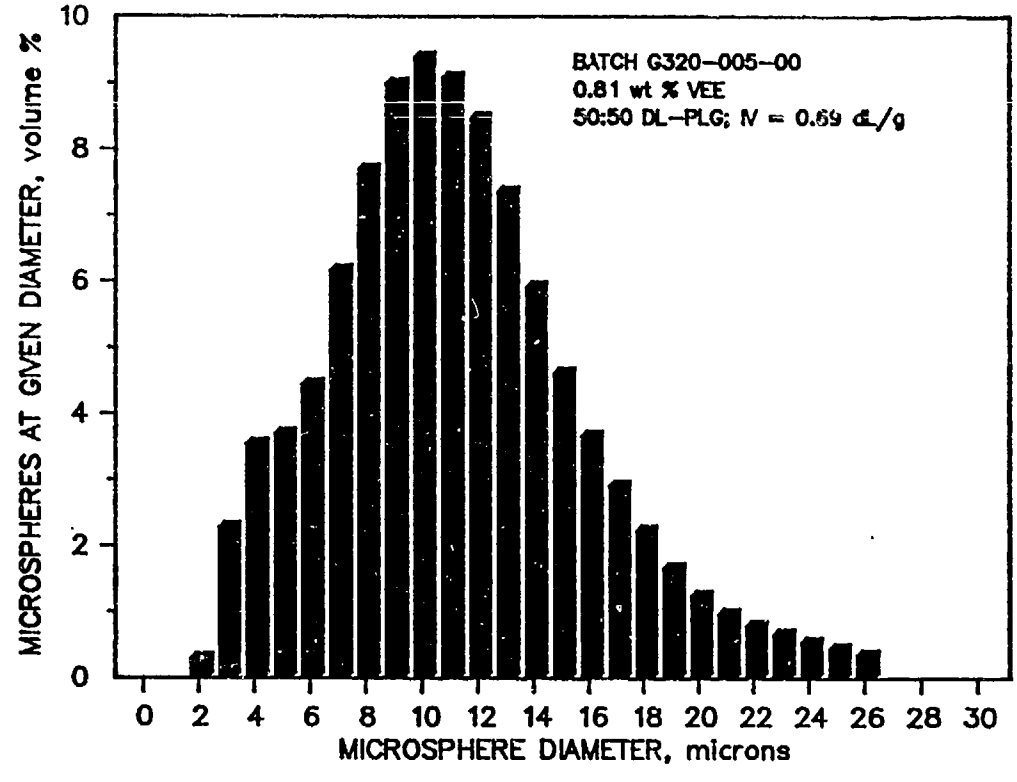


Figure 1. Cumulative in vitro release of VEE (above) and size distribution (bottom) data: Batch G320-005-00.

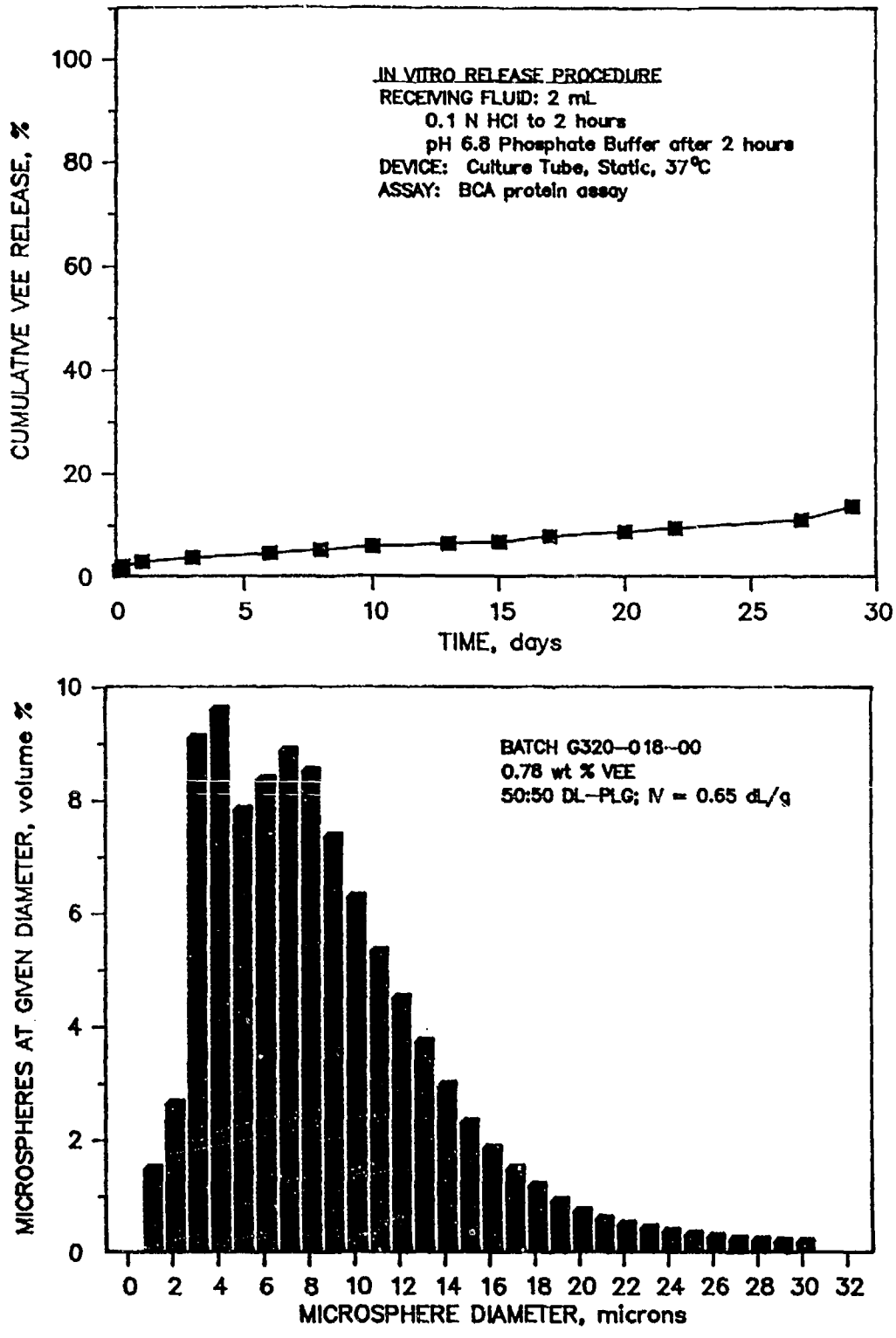


Figure 2 Cumulative in vitro release of VEE (above) and size distribution (bottom) data: Batch G320-018-00.

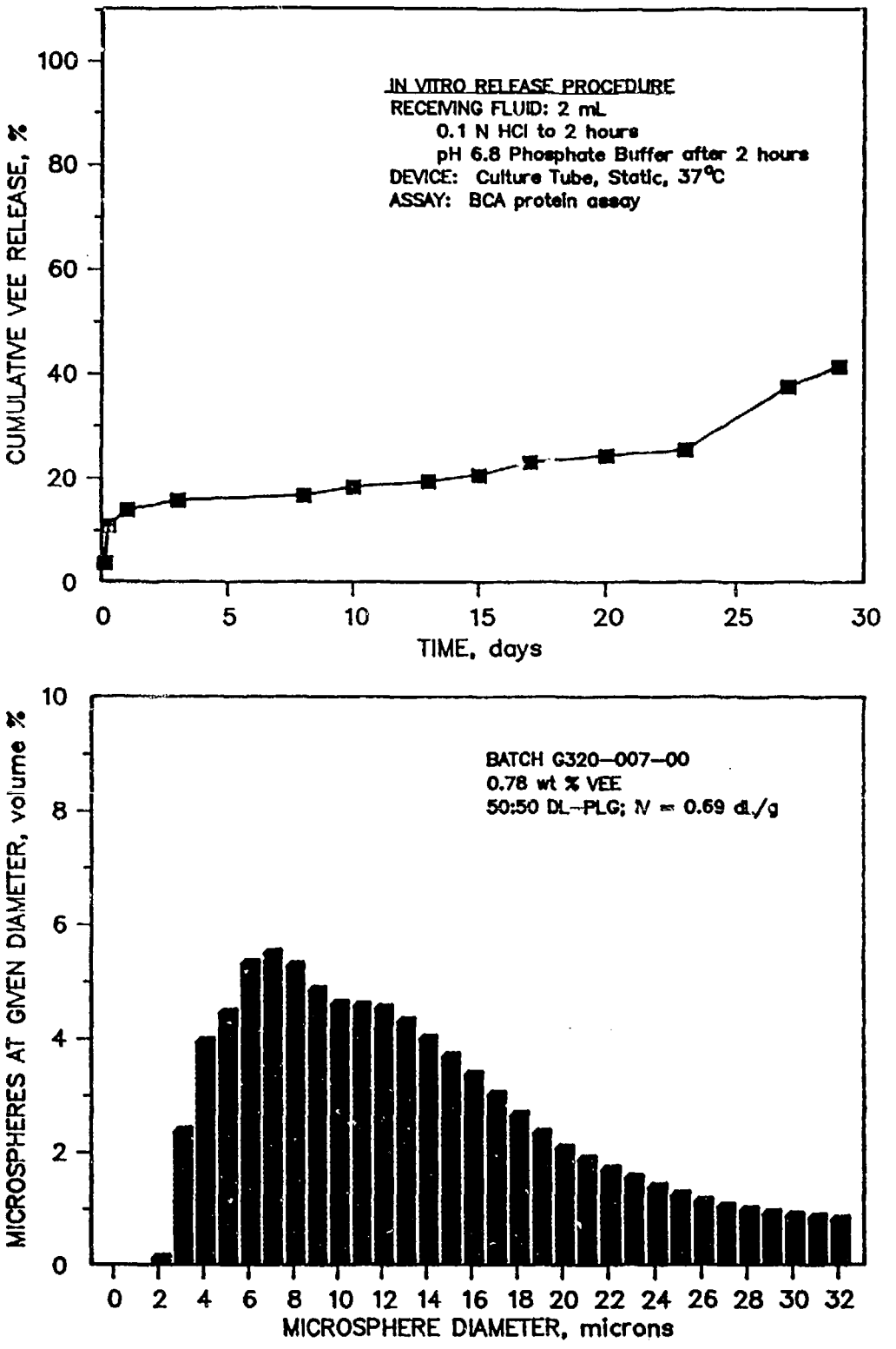


Figure 3 Cumulative in vitro release of VEE (above) and size distribution (bottom) data: Batch 6320-007-00.

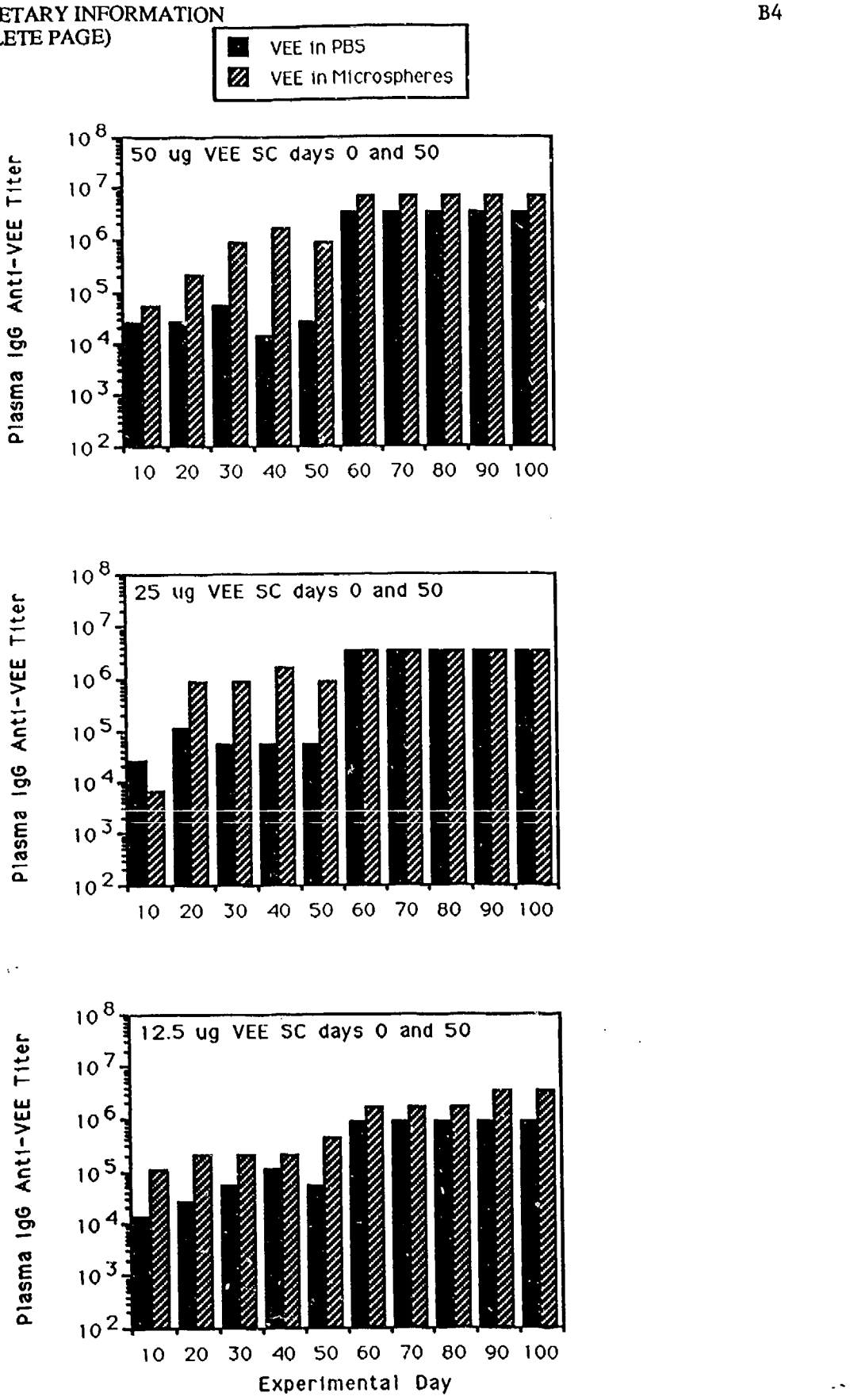


Figure 4. Plasma IgG anti-VEE virus antibody response in mice immunized with various doses of microencapsulated and nonmicroencapsulated formalinized TC 83 whole VEE virus vaccine. Groups of SPF BALB/c mice were immunized by the SC injection on days 0 and 50 of the indicated dose of VEE virus vaccine in PBS or encapsulated in 50:50 DL-PLG microspheres. (Batch G320-005-00 methylene chloride process solvent). Plasma samples obtained at the indicated times were assayed for IgG anti-VEE antibodies by ELISA.

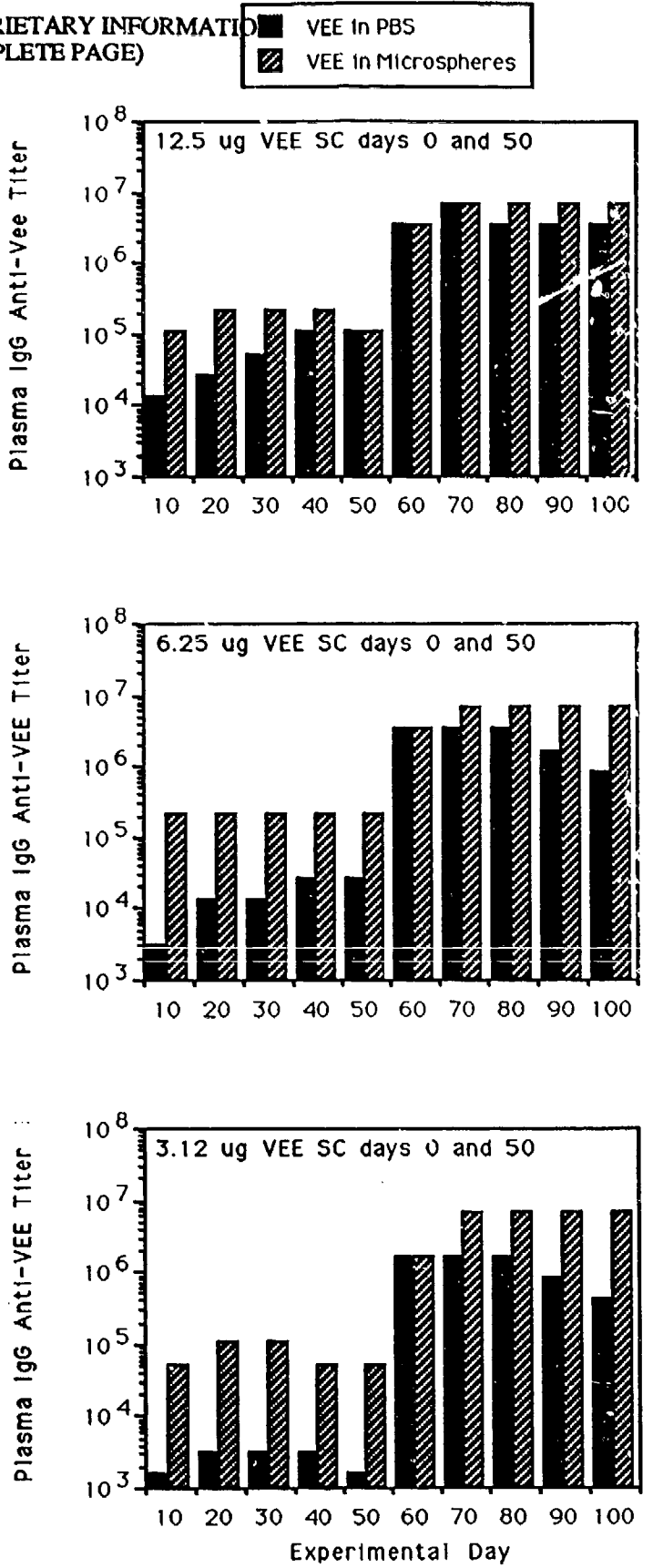


Figure 5. Plasma IgG anti-VEE virus antibody response in mice immunized with various doses of microencapsulated and nonmicroencapsulated formalinized TC 83 whole VEE virus vaccine. Groups of SPF BALB/c mice were immunized by the SC injection on days 0 and 50 of the indicated dose of VEE virus vaccine in PBS or encapsulated in 50:50 DL-PLG microspheres. (Batch G320-018-00 methylene chloride process solvent). Plasma samples obtained at the indicated times were assayed for IgG anti-VEE antibodies by ELISA.

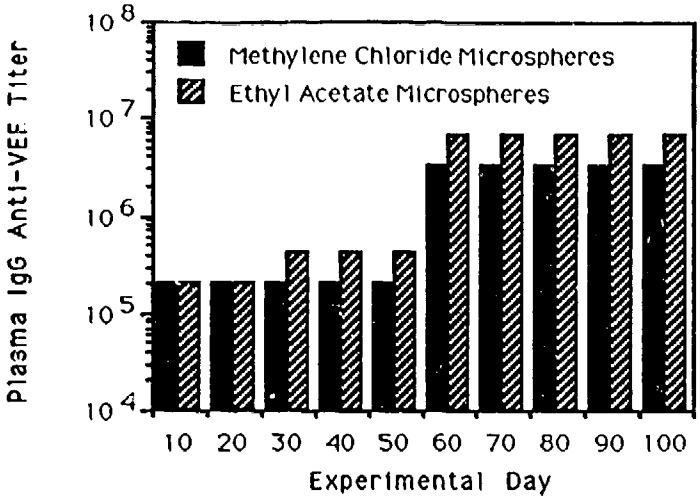


Figure 6. Plasma IgG anti-VEE virus antibody response in mice immunized with 25 µg of formalinized TC 83 whole VEE virus vaccine encapsulated in 50:50 DL-PLG microspheres prepared using either methylene chloride (Batch G320-018-00) or ethyl acetate (Batch G320-007-00) as the process solvent. Groups of SPF BALB/c mice were immunized by the SC injection of the microencapsulated vaccine on experimental days 0 and 50. Plasma samples were obtained at the indicated times and assayed for IgG anti-VEE virus antibodies by ELISA.

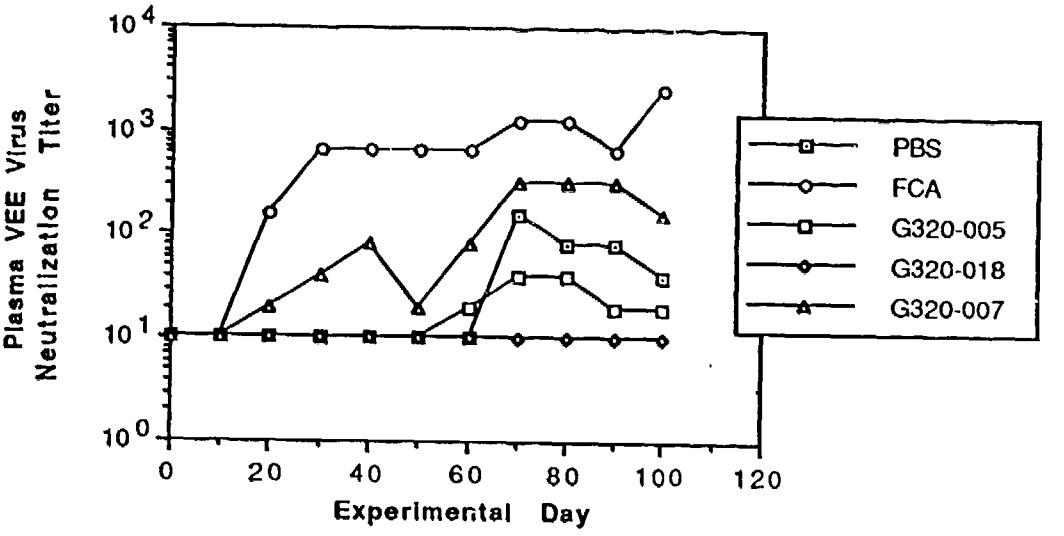


Figure 7. VEE virus neutralizing activity in the plasma of mice immunized with 25 µg of formalinized TC 83 whole VEE virus vaccine in different vehicles. Groups of SPF BALB/c mice were immunized on experimental days 0 and 50 by the SC injection of the vaccine in PBS, emulsified in complete Freund's adjuvant, or encapsulated in 50:50 DL-PLG microspheres manufactured using methylene chloride (Batches G320-005, G320-018) or ethyl acetate (Batch G320-007) as the process solvent. Plasma samples were obtained at the indicated times and assayed *in vitro* for anti-VEE virus neutralizing antibodies.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 21 Apr 97

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCP, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for Contract Number DAMD17-90-C-0113. Request the limited distribution statement for Accession Document Number ADB164489 be changed to "Approved for public release; distribution unlimited." This report should be released to the National Technical Information Service.

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Colonel, MS
Deputy Chief of Staff for
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