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Project 6160-XXV

DEVELOPMENT OF NEW IMMUNOGENS AND A CONTROLLED RELEASE DELIVERY SYSTEM FOR ORAL IMMUNIZATION AGAINST STAPHYLOCOCCAL ENTEROTOXIN B

Annual Report

Jay K. Staas, John H. Eldridge, Richard M. Gilley, Thomas R. Tice

December 6, 1991

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13. ABSTRACT *(Maximum 200 words)*

During this reporting period, experiments were performed in which rhesus monkeys were immunized with an SEB toxoid microsphere vaccine delivery system. The immune responses were followed and the monkeys ultimately received a challenge with lethal doses of aerosolized SEB toxin.

The vaccine delivery system consisted of SEB toxoid microencapsulated in a 50:50 poly(DL-lactide-co-glycolide) excipient. The immunization schedule consisted of the nine possible combinations of intramuscular (IM), oral, and intratracheal (IT) primary and secondary immunizations. Primary immunizations were given on Day 0 and secondary immunizations were administered on Day 49.

Two of the four planned replicate monkey experiments were concluded during this reporting period. The monkey antibody levels suggested that an IM primary followed by and IT or oral secondary immunization would provide the highest degree of protection.

For the first time, the ability to protect monkeys against an aerosol challenge with a lethal dose of SEB toxin was documented. The monkeys that received an IM primary immunization followed by an IT secondary immunization survived. In addition, the monkey from the first experiment, EX-Rh-101, that received the oral/IT immunization regimen survived as did the monkey that had been given the IT/IT immunization regimen in the second experiment.

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DEVELOPMENT OF NEW IMMUNOGENS AND CONTROLLED RELEASE DELIVERY SYSTEM FOR IMMUNIZATION AGAINST STAPHYLOCOCCAL ENTEROTOXIN B

I. EXECUTIVE SUMMARY

During this year of the contract, we obtained a great deal of knowledge about the immunization of rhesus monkeys with SEB toxoid. A preliminary study was performed to confirm the existing data regarding the effectiveness of intramuscular (IM) immunization with alum precipitated SEB toxoid and oral immunization with high doses of toxoid in solution, to raise circulating anti-SEB toxin antibodies. Additionally, several studies involving the immunization of rhesus monkeys with SEB toxoid microspheres have also been performed.

The preliminary monkey experiment yielded the following results: Immunization with alum-precipitated SEB toxoid did result in the appearance of circulating IgG antibodies, which were detected in low levels in the BAW fluids. Oral immunization with SEB toxoid solution induced only the transient appearance of low levels of IgM and IgA anti-SEB toxin antibodies in circulation.

Results from the first two studies (EX-Rh-101 and EX-Rh-102) in which rhesus monkeys were immunized with microencapsulated SEB toxoid are included in this report. These data suggest that an IM primary immunization followed by an intratracheal (IT) or oral secondary immunization provides the highest antibody response and best level of protection. It has been reported to us that the monkeys receiving an IM primary and IT secondary immunization in both studies survived aerosol challenge. In addition, the monkey receiving an oral primary and IT secondary immunization from Study EX-Rh-101 and the monkey receiving both an IT primary and IT secondary immunization in Study EX-Rh-102 also survived the aerosol challenge. Official documentation of these results has not been received by Southern Research or the University of Alabama in Birmingham.

These results are very encouraging as not only have high antibody titers been achieved in the rhesus monkeys, but as indicated above, several of the monkeys have been shown to be protected from aerosol challenge.

II. STATEMENT OF THE PROBLEM UNDER STUDY

The staphylococcal enterotoxins are extracellular proteins produced by Staphylococcal aureus that have been demonstrated to be a major cause of food poisoning. Serological methods have differentiated five classes of enterotoxins, A, B, C1, C2, D, and E (1-6) (Note: References can be found in Section VII, see Page 22). Despite extensive study of the structure and properties of these enterotoxins, their precise mode of action is

unclear (7). While detection of the toxins and prophylaxis of toxemia is of obvious importance to the food industry and medical community, there is also significant concern from a military perspective about the potential use of staphylococcal enterotoxins as biological-warfare agents.

Over the last 20 years, studies at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) on the pathophysiological effects of staphylococcal enterotoxin B (SEB) have demonstrated its lethal toxicity at low doses (25 μg/kg body weight) in rhesus monkeys (8), with presumably similar effects in man. Studies by Liu et al. (9, 10, 11) have shown that SEB administered intravenously to rhesus monkeys in high doses (1 mg/kg body weight) results in death within 20 hours due to pulmonary dysfunction and edema, with secondary effects on cardiovascular, hepatic, and renal function. Under a biological-warfare scenario, military personnel could be exposed to large quantities of SEB, probably in an aerosol form, which might result in lethal toxemia more rapidly than similar intravenous doses, given the sensitivity of the lungs to the toxin. Immunization with toxoids of SEB that result in high levels of neutralizing antibodies might be an effective and inexpensive defense against possible exposure to SEB by military personnel.

The objective of this research program is to develop an SEB toxoid microsphere formulation with biocompatible, biodegradable polymers. After oral administration of these microspheres, the microsphere formulation will be designed to target and control the release of SEB toxoid in the Peyer's patches, so as to elicit a protective secretory immune response.

III. BACKGROUND AND REVIEW OF THE LITERATURE

A. Secretory IgA

The mucosal surfaces of man and other mammals are in direct continuity with the external environment and represent the major bodily site of antigenic exposure and recognition. Mucosal secretions covering these tissues represent a major host defense mechanism, which is often times underestimated in importance. It was recognized only 20 years ago that large differences exist in the proportions of the various immunoglobulin classes present in external secretions as opposed to serum. The discovery by Tomasi and colleagues (12, 13) and Hanson (14) that IgA is the major isotype in human and other mammalian external secretions provided the impulse for numerous studies on the mucosal immune system in both health and disease. The immunoglobulins secreted at these sites are primarily produced by local plasma cells which heavily infiltrate the lamina propria regions of mucosal sites such as the gut, nasal passage and salivary glands.

Secretory IgA (sIgA) antibodies are structurally and functionally distinct from the immunoglobulins which make up the recirculating pool in serum. sIgA exists primarily as a dimer in association with a molecule of secretory component (70,000 daltons) and a molecule of J Chain (15,000 daltons). The covalently bound secretory component is hypothesized to wind around the Fc portions of the two IgA molecules and, through secondary

interactions, stabilize the IgA molecules against proteolytic cleavage. This structure may provide a distinct advantage in the efficacy of sIgA in the gut and oral cavities, which commonly contain bacteria that produce proteolytic exoenzymes. IgA is neither opsonic nor does it fix complement (both characteristics of IgM and most classes of IgG antibodies), but it is highly effective in viral and toxin neutralization (15, 16) and in inhibiting the adherence of bacteria to epithelial cell surfaces (17). The unique characteristics of sIgA are doubtlessly a reflection of the need for this immunoglobulin to function outside the body where it prevents antigen adherence and penetration.

B. Antibodies in the Respiratory Tract

Antibodies present within the respiratory tract originate from two different sources. IgA predominates in the mucus which bathes the nasopharynx and bronchial tree (18, 19). More than 90% of this IgA is in the 11S-dimeric form and has attached secretory component and J chain, while only a small amount is in the 7S-monomeric form (20). This distribution is in contrast to the serum IgA of humans, which is virtually all monomeric. The molecular weight of sIgA 390,000-395,000 daltons is well above the approximately 200,000-dalton cutoff imposed by the capillary-alveolar membrane, and the IgA/albumin ratio in the bronchial-alveolar wash (BAW) fluids is substantially higher than in serum, indicating that the bulk of bronchial IgA is locally produced (21). In this regard, IgA plasma cells have been shown to line the lamina propria of the airway wall and are particularly concentrated about the bronchial glands (18). Dimeric IgA from these plasma cells is bound to secretory component expressed on the basal surface of bronchial epithelial cells and reaches the external secretions via active transport through this epithelium (22). In contrast to the nasopharynx and bronchial tree, the bronchioli and alveoli predominantly contain IgG (23). The ability of IgG (158,000 daltons) to freely pass the capillary-alveolar membrane, plus the fact that the IgG/albumin ratio in BAW fluids is the same as that in serum, indicates the bulk of pulmonary IgG is passively derived from the intravascular pool (21, 23).

C. Gut-Associated Lymphoreticular Tissue and the Common Mucosal Immune System

The finding by Craig and Cebra (24) that Peyer's patches which are distinct lymphoreticular follicles along the gastrointestinal tract (GI tract) possess IgA precursor B cells which can repopulate the lamina propria regions of the gastrointestinal and upper respiratory tracts and differentiate into mature IgA synthesizing plasma cells, suggested that the induction of IgA responses is not necessarily a local phenomenon. The Peyer's patches contain a large subpopulation of B lymphocytes that are committed to IgA synthesis, all categories of regulatory T lymphocytes, and functional accessory cells, i.e., macrophages and dendritic cells (25, 26). In addition, Peyer's patches are covered by a unique epithelium which contains microfold cells (M cells) with highly developed pinocytotic channels that allow sampling of antigens from the gut lumen, and transport to cells in the underlying dome region, with subsequent stimulation in the B cell (follicles) and T cell (parafollicular) zones.

Heremans and Bazin et al. (27, 28) measured the development of IgA responses in mice orally immunized with soluble (28) or particulate (27) antigen. A sequential appearance of antigen-specific IgA lymphoblasts occurred, first in mesenteric lymph nodes, later in the spleen, and finally in the lamina propria of the GI tract. Subsequent studies have shown that oral administration of antigen leads to production of sIgA antibodies in the gut and also in secretions distant to the gut, e.g., in bronchial washings, colostrum, milk, saliva, and tears (29-32). Coupled with the findings of Craig and Cebra (24), these results suggested that Peyer's patches are enriched sources of precursor IgA B cells, which, subsequent to antigen sensitization, follow a circular migrational pathway and account for the expression of IgA at distant mucosal surfaces. This circular traffic pattern provides a common mucosal immune system by continually shuttling sensitized B cells to mucosal sites for responses to gut-encountered environmental antigens and potential pathogens (33). This circulatory pathway helps explain the presence of naturally occurring IgA antibodies to microorganisms at sites where local antigenic stimulation would not be expected to occur. This is best exemplified by the presence of sIgA antibodies in human colostrum to gut Escherichia coli (32) and the oral pathogen Streptococcus mutans (34).

Of particular importance to this project is the ability of oral immunization to induce protective antibodies in the respiratory tract. In this regard, studies have demonstrated that the ingestion of various antigens by humans (35, 36), primates (37), rabbits (38), rats (39), and mice (36) results in the appearance of antigen-specific sIgA antibodies in bronchial and/or nasal washings. Experiments by Waldman and colleagues (36) have shown that when mice are immunized with live influenza virus via the nasal, oral, or rectal routes, protection against aerosol challenge is conferred. The protection correlated with the level of antibodies secreted into the respiratory tract, but no correlation with serum antibody levels was found. In contrast, parenteral immunization was found to be a good method to induce humoral antibodies against influenza, but a poor method to induce pulmonary antibodies and was non-protective. Extension of these studies with human volunteers confirmed that oral administration of influenza vaccine was effective at inducing secretory anti-influenza antibodies in nasal secretions (40).

Several investigations have shown that secretory antibody levels in humans (41, 42) and mice (43, 44) correlate with protection against pulmonary viral infection to a significantly greater extent that do circulating antibody titers. This offers a valid explanation for the observation made over 50 years ago by Bull and McKee (45) that intranasal immunization of rabbits with killed pneumococci resulted in resistance to pulmonary challenge with live homologous pneumococci in the absence of circulating antibodies. Therefore, it appears that oral immunization to stimulate the pulmonary immune system may have many advantages, including effectiveness, safety, decreased side effects, and the potential for an almost unlimited number and frequency of boostings.

IV. RATIONALE USED IN THE CURRENT STUDY

The use of microencapsulation to protect sensitive bioactive agents from degradation and to control their release over extended periods of time in vivo has become quite prevalent. The technique involves the coating of a bioactive agent (solid or liquid) with a protective wall material. The wall materials are usually polymeric in nature. The microsphere product is a free-flowing powder of spherical particles. The agent to be encapsulated can be coated with a single wall of the polymeric material, or it can be homogeneously dispersed within a polymeric matrix. The amount of agent inside the microspheres can be very small or can range to as high as 95% of the microsphere composition. The diameter of microspheres can be less that 1 μm or as large as 3 mm.

The use of microspheres to deliver vaccine antigens to the Peyer's patches offers several advantages. First, a microsphere formulation can be designed to protect the antigens from degradation during passage through the gastrointestinal tract and then facilitate uptake into the Peyer's patches. After uptake, the microspheres can release the vaccine antigens at a controlled rate over a period of hours to months.

One microsphere system of particular interest involves the use of poly(lactide-co-glycolide)s (PLGs) (46-47). PLGs are biocompatible, biodegradable polyesters and are from the same class of material used in resorbable sutures. They biodegrade in vivo into lactic acid and glycolic acid, eventually carbon dioxide and water. The mechanism of degradation is by hydrolysis of the ester linkages. The rate of degradation for these copolymers is primarily determined by the ratio of lactide to glycolide in the copolymer (58). For instance, DL-PLG with a 50:50 mole ratio of DL-lactide-to-glycolide will completely biodegrade in vivo when administered subcutaneously (SC) or intramuscularly (IM) within about 6 weeks, while poly(DL-lactide)(DL-PL) completely biodegrades in about 10 to 12 months.

One of the major advantages of DL-PLG microsphere systems is the flexibility allowed in formulating the specific duration of release. It is well known that specific antigens require different lengths of exposure to elicit a strong primary response. The time required before re-exposure to elicit the most potent secondary response also varies with different antigens. By using different combinations of DL-PLGs, delivery systems that demonstrate the proper release rates or release program can be prepared for virtually any antigen. More specifically, a formulation can be designed so that part of the antigen is released soon after the microspheres are taken up by the PP to elicit the primary response. Then, no additional antigen will be released from the microspheres until sufficient time has elapsed to obtain an efficacious secondary response. At this time (as a result of degradation of the polymer), additional antigen will be released, potentiating the secondary response. If desired, multiple releases of antigen at different times could be incorporated into the final microsphere formulation. The times at which the antigen would be released from the formulation would depend on the DL-PLGs used in the formulation.

V. EXPERIMENTAL METHODS

A. Mice

BALB/c (original breeders obtained from the Jackson Laboratories, Bar Harbour, ME) were bred and maintained in our facilities at the University of Alabama at Birmingham. All mice used in these studies were 8 to 12 weeks of age at the initiation of the procedures and were of mixed sexes.

B. Rhesus Monkey

Macaqua mulatta monkeys were obtained from the Texas Primate Center. All monkeys were 9 to 12 months of age upon receipt.

C. Immunologic Reagents

Solid-phase absorbed and affinity-purified goat IgG antibodies specific for murine IgM, IgG, and IgA were obtained from (Southern Biotechnology Associates, Birmingham, AL). Their specificity was confirmed in radioimmunometric assays (RIA) using purified monoclonal antibodies and myeloma proteins as substrates. Hybridoma cell lines producing monoclonal antibodies specific for the murine antigens Thy 1.2 [30-H12, rat IgG_{2b} (59)], Ly-1 [53-7.313, rat IgG_{2a} (59)], Lyt-2 [53-6.72, rat IgG_{2a} (59)], L3T4 [GK 1.5, rat IgG_{2b} (60)], B 220 [RA 3-3A1/6.1, rat IgM (61)], IgM [331.12, rat IgG_{2b} (62)] and MAC-1 [M1/70.15.11.5, rat IgG_{2b} (63)] were obtained from the American Type Culture Collection, (Rockville, MD). All lines were propagated in vitro and the antibodies in the culture supernatants were purified by sequential precipitation at 50% saturation in ammonium sulphate, anion-exchange chromatography (DE-52, Whatman, Kent, England), and sizing on AcA 34 (LKB, Bromma, Sweden) for IgG and AcA 22 for IgM.

D. Radioiodination and Immunoradiometric Assays

Solid-phase absorbed and affinity-purified goat IgG antibodies specific for mouse total immunoglobulins, IgM, IgG and IgA were radioiodinated with carrier-free Na ^{125}I (Amersham, Arlington Heights, IL) using the chloramine-T method modified to reduce oxidative damage to proteins (64). Radioimmunometric assays (RIA) were performed in Immulon assay strips (Dynatech, Chantilly, VA) coated with SEB toxoid at 5 μg/mL in pH 8.4, borate-buffered saline (BBS) overnight at 4 °C. Control strips were left uncoated, but all strips were blocked for 2 h at room temperature with 1% (w/v) bovine serum albumin (Sigma, St. Louis, MO) in BBS, which was also used as the diluent for all samples and ^{125}I-labeled reagents. Various 2-fold dilutions of test sera were added to washed triplicate

replicate wells and incubated for 6 h at 25 °C. After washing, 100,000 cpm of ^{125}I-labeled isotype-specific anti-immunoglobulin reagent was added to each well and incubated overnight at 4 °C. Following removal of the unbound ^{125}I-antibodies by washing, the wells were counted in a Gamma 5500 spectrometer (Beckman Instruments, Irvine, CA). The results have been presented as the reciprocal of the serum dilution producing a signal greater than 3 times that of the group-matched prebleed at the same dilution (end-point titration).

E. Characterization of Microspheres

The core loading for each batch of microspheres was determined by first extracting the protein (SEB toxoid) from a known amount of microspheres. The extracted protein was then quantified using a colorimetric protein assay. The antigen-containing microspheres were also characterized with respect to in vitro release kinetics using the procedures outlined in our First Annual Report, Pages 8 to 10.

F. Procedure for Sampling Rhesus Monkey Lung Secretions

As the major goal of the rhesus studies is to investigate the induction of SEB toxin-neutralizing antibodies in the lungs, the first group of primates was used to develop the procedures for collecting samples of pulmonary secretions. In order for these samples to be useful in the evaluation of the various immunization schemes to be tested under this contract effort, they must contain a level of total immunoglobulin which is sufficient to allow detection of the antigen-specific component. Further, antibodies of the IgG and IgA isotypes must be well represented in order to draw firm conclusions about the relative contribution of locally synthesized versus blood circulation derived responses.

Initial attempts to obtain secretions using a pediatric bronchoscope were unsuccessful. The internal tracheal diameter of the young rhesus monkeys was found to be unexpectedly small by comparison to a human infant of comparable weight. The result was that the 4-mm outer diameter of the bronchoscope completely occluded the trachea upon insertion. Although samples could be obtained from the proximal airway by lavage, they were of insufficient volume and antibody content to be of use. In addition, the monkeys rapidly became cyanotic, necessitating that the work be performed over such a short period of time as to prevent careful and reproducible sample collection.

To overcome the above limitations the sampling procedure was changed to one which employs the insertion of a tracheal tube, through which a suction catheter may be passed. This approach has proven to be highly successful, and has been adopted for these primate studies. In brief, fasted monkeys were anesthetized by the intramuscular injection of ketamine hydrochloride. After obtaining a blood sample, and gastric contents were removed by suction through an 8-French x 14-inch pediatric feeding tube inserted into the stomach. Intratracheal intubation was accomplished with a 3-French x 12-cm pediatric tracheal tube which was inserted through the

glottis with the aid of a 6-French intubating stylet. After taping the tracheal tube in place, the animal was briefly ventilated with humidified 100% oxygen to prevent hypoxemia during the subsequent steps. One mL of phosphate buffered saline (PBS) was placed into the hub of the tracheal tube and allowed to pass into the lungs by normal respiratory action. An 8-French x 14-inch suction catheter, attached to a vacuum line through a 15-mL trap, was immediately passed through the tracheal tube to recover the wash fluid. The suction catheter was then withdrawn and the mucus adhering to the bore of the tube was washed into the trap with 2 mL of additional PBS. The samples were clarified by centrifugation. Sodium azide, phenylmethyl-sulfonyl fluoride, and fetal calf serum were added as preservative, protease inhibitor, and alternate substrate for protease activity, respectively. All samples were stored at -70 °C until assayed.

VI. RESULTS

A. Preparation of SEB Toxoid Microspheres

During this reporting period, we prepared the SEB toxoid microspheres for use in the rhesus monkey studies. The initial batch of microspheres (Composite F787-074-00), that we prepared were too small (97.3% < 5.3 μm in diameter). The in vitro release profile also indicated too rapid a release of the SEB toxoid. These data are shown in Figure 1 (Appendix B). A second composite batch of microspheres was then prepared, F787-110-00. This batch consisted of microspheres that were more appropriately sized and had more favorable in vitro release characteristics. These data are illustrated in Figure 2.

One final batch of SEB toxoid microspheres was prepared. This batch (Composite F787-124-00), was prepared to ensure that a sufficient supply of microspheres would be on hand for the monkey studies. The in vitro release profile and size distribution for this batch of microspheres are shown in Figure 3.

All of the SEB toxoid microspheres prepared during this reporting period are described in Table 1. Size distributions for these microspheres are summarized in Table 2.

B. Immunization Results of Monkey Study EX-Rh-100

The preliminary rhesus monkey study, EX-Rh-100, was designed to provide a bridge to the existing data regarding the ability of IM immunization with alum-precipitated SEB toxoid and oral immunization with high doses of toxoid in solution to raise circulating anti-SEB toxin antibodies. This experiment also provides information about the levels of anti-toxin in lung secretions after immunization by these two methods. The toxoid forms, doses and the timing of the doses were selected to approximate the studies of Bergdoll (37).

For Study EX-Rh-100, three groups of two monkeys each were immunized as follows:

Group 1: Monkeys 89 B109 and 89 B093
Normal controls

Group 2: Monkeys 89 B106 and 89 B115
100 µg of SEB toxoid on alum, administered by IM injection on Days 0, 49, and 105.

Group 3: Monkeys 89 B043 and 89 B103
10 mg of SEB toxoid in 0.7% bicarbonate, administered by oral gavage on Days 0, 49, and 105.

Plasma samples were collected on Day 0 and at 7-day intervals through Day 98. BAW samples were collected on Day 0, 28, 49, 77, and 98.

1. Plasma anti-SEB toxin responses

End-point titration in ELISAs employing solid-phase adsorbed SEB toxin confirmed the absence of toxin-reactive antibodies of any isotype in the prebleeds of all the monkeys employed in this study. The control monkeys, 89 B109 (Figure 4) and 89 B093 (Figure 5), remained serologically negative in all isotypes to SEB toxin at the lowest tested dilution of plasma (1:50) throughout the period of this study.

The two monkeys immunized with SEB toxoid precipitated on an alum slurry, 89 B106 (Figure 6) and 89 B115 (Figure 7), responded with the production of SEB toxin-specific circulating antibodies in each of the three isotypes. IgM anti-SEB toxin titers reached maximal levels 14 days after the primary immunization in both monkeys, and were detectable throughout the primary responses. Following boosting on Day 49, Monkey 89 B115 demonstrated a secondary rise in IgM anti-toxin, while Monkey 89 B106 did not. However, late in the course of the secondary response, IgM anti-toxin titers in both monkeys became undetectable. The bulk of the circulating anti-SEB toxin responses in the monkeys receiving alum precipitated toxoid were of the IgG class. Maximal IgG anti-SEB toxin titers were achieved in both monkeys by Day 28. Monkey 89 B106 mounted an earlier and more vigorous IgG response which reached a peak titer of 102,400 on Day 21, while Monkey 89 B115 exhibited a peak titer of 25,600 which was not reached until Day 28. However, both monkeys exhibited a secondary response which achieved a maximal titer of 409,600 on the 14th day following boosting. Circulating IgA anti-SEB toxin antibodies were only observed in low titers after immunization with the alum-precipitated toxoid. One monkey, 89 B106, produced a low and transient IgA response which coincided with the peak of the secondary response. The other monkey, 89 B115, produced a low IgA response across the later portion of the primary response, but no response was detectable after boosting. Thus, the two monkeys injected with alum-precipitated toxoid responded somewhat differently to the primary immunization. Monkey 89 B106 responded more rapidly and produced 4- to 8-fold higher levels of IgM and IgG anti-toxin than Monkey 89 B115. However, following secondary immunization, both monkeys achieved the same circulating titer of IgG anti-toxin.

In contrast to the high titers of circulating anti-toxin antibodies achieved by the systemic injection of 100-μg doses of toxoid on alum, the two monkeys receiving 10-mg doses of toxoid orally exhibited only sporadically detectable circulating anti-toxin antibodies. As shown in Figure 8, Monkey 89 B043 produced low, but clearly detectable, IgA anti-toxin antibodies on Day 28 after the primary administration and 7 days after the secondary administration. Monkey 89 B103 also exhibited a low circulating IgA response (Figure 9) 7 days after the secondary administration and an additional transient IgM response on Day 35 and 42 following the secondary administration. Overall, the administration of even high doses of SEB toxoid in solution appears to have been virtually ineffective at the induction of circulating anti-SEB toxin antibodies.

2. Bronchial-alveolar wash anti-SEB toxin responses

Assays of the BAW fluids obtained from the monkeys in EX-Rh-100 revealed that SEB-specific antibodies could only be detected in the samples of the lung fluids obtained from the two monkeys which had received IM immunization with the alum-precipitated toxoid (Table 3). These antibodies were restricted to the IgG isotype and were not detected until Day 49. They were present at the highest level (titers of 80) on Day 77, and fell to titers of 10 on Day 98. Following a tertiary IM immunization on Day 105, these monkeys resisted aerosol challenge with SEB toxin.

C. Immunization Results of Monkey Study EX-Rh-101

Experiment EX-Rh-101 was designed to investigate the efficacy of immunizing with SEB toxoid microspheres. More specifically, 10 monkeys were employed in testing SEB toxoid microspheres prepared with a 53:47 DL-PLG excipient. These microspheres, composite Batch F787-110-00, contained 0.41 wt % SEB toxoid and were 1 to 10 μm in diameter. One of the 10 monkeys was used as a nonimmunized control, and the remaining 9 monkeys were administered SEB toxoid microspheres in the nine possible combinations of primary and secondary immunizations using the IM, oral and intratracheal (IT) routes of administration. A summary of the immunization regimens is given in Table 4.

The rhesus monkeys were bled for plasma samples on Day 0, and every 7 days thereafter through Day 98. BAW samples were obtained on Days 0, 28, 49, 77, and 98. All samples were tested for SEB toxin specific antibodies of the IgM, IgG, and IgA isotypes by end-point titration.

1. Plasma anti-SEB toxin responses following primary immunizations

The nonimmunized control monkey (89 B088) did not exhibit detectable plasma anti-SEB toxin antibodies of any isotype at any time point tested (Figure 10).

a. Primary IM immunization (Monkeys 89 B096, 89 B161, and 89 B210)

Each of the monkeys that were administered a primary IM immunization with 100 μg of microencapsulated SEB toxoid mounted a brisk circulating anti-toxin response which was observed in all isotypes, but which was predominantly IgG. These immune responses are graphically illustrated in Appendix B as indicated:

Monkey	Figure
89 B096	11
89 B161	12
89 B210	13

The responses by Monkeys 89 B161 and 89 B210 were similar in that their maximal primary IgG anti-toxin titers of 102,400 were attained on Day 28 and remained at this level through Day 49 when booster immunizations were administered. In contrast, Monkey 89 B096 mounted a plasma IgG response which arose more slowly, but which steadily increased through Day 49 to a titer of 409,600. Thus, each of the monkeys immunized with 100 μg of encapsulated SEB toxoid attained primary IgG anti-toxin levels which equalled or exceeded the peak primary IgG anti-toxin titer reached by the monkeys making the highest response to immunization with 100 μg of SEB toxoid precipitated on alum. In addition, the monkeys immunized with the microencapsulated toxoid maintained their anti-toxin levels better, and by Day 49 their plasma IgG anti-toxin titers were 2 to 16 times higher than the monkeys immunized with the alum-precipitated toxoid.

b. Primary IT immunization (Monkeys 89 B079, 89 B001, and 89 B116)

Primary IT immunization with 100 μg of microencapsulated SEB toxoid induced a circulating anti-SEB toxin response in each of the three monkeys. These responses are given in Appendix B as follows:

Monkey	Figure
89 B079	14
89 B001	15
89 B116	16

In two of the three monkeys (89 B079 and 89 B001), the response was exclusively of the IgM isotype. The third monkey, 89 B116, produced an early IgM response which was followed by an IgA response. The IgM responses were quite individual in nature: Monkey 89 B079 produced an early, high response, Monkey 89 B001 produced a late response of intermediate level, and Monkey 89 B116 produced an early but low response. None of the IT-immunized monkeys produced a measurable plasma IgG anti-toxin response at any of the time points examined.

Note: The complete absence of a circulating IgG response, and the presence of only a low IgA response in one of the monkeys, is in contrast to the extremely vigorous responses observed in mice following IT immunization with SEB toxoid microspheres (reference Third Annual Report, page 12). This prompted us to review the manner in which the primary IT immunization of these three monkeys had been performed. After review, we realized that the microsphere suspension had been instilled through a catheter passed deep into one lung. This likely delivered the microspheres to only one segment of one lobe. In contrast, the IT immunizations employed in the mouse studies involved the instillation of the suspension into the trachea. This technique resulted in a more uniform deposition in both the upper and lower respiratory tract. All subsequent IT immunizations of the rhesus monkeys (beginning with the EX-Rh-101 booster immunizations) were performed by passing the catheter just beyond the end of the intratracheal tube. Significantly improved responses have resulted, as shown in the EX-Rh-101 monkeys receiving secondary immunizations via the IT route (data discussed below).

c. Primary oral immunization (Monkeys 89 B060, 89 B064, 89 B034)

Following oral immunization with the microencapsulated SEB toxoid, one of the monkeys (89 B060) did not exhibit a demonstrable plasma anti-toxin response. However, the other two monkeys (89 B064 and 89 B034) did produce circulating antibodies. The responses are illustrated in Appendix B as indicated:

Monkey	Figure
89 B060	17
89 B064	18
89 B034	19

Monkey 89 B034 produced an IgM response which was present at a titer of 800 on Days 28 through 49. In contrast, Monkey 89 B064 responded with an early IgM response which peaked on Day 14 and fell to an undetectable level on Day 42. In addition, this monkey produced an IgG anti-toxin response beginning on Day 28 and peaking on Day 4 at a titer of 1,600. Although 2 of the 3 monkeys immunized with the microencapsulated SEB toxoid produced clear responses in the plasma, none exhibited detectable IgA anti-toxin activity.

2. Plasma anti-SEB toxin responses following secondary immunizations

a. Secondary immunizations of IM-primed monkeys

As a group, the monkeys administered primary IM immunizations with microencapsulated toxoid, and boosted with the SEB toxoid microspheres either systemically or by the two mucosal routes, exhibited good levels of circulating anti-toxin antibodies. The orally-boosted monkey (89 B096, Figure 11) maintained an IgG antibody level which was of the same order of magnitude as monkeys administered primary and secondary immunizations with toxoid precipitated on alum. The IT-boosted monkey (89 B161, Figure 12)

and the IM-boosted monkey (ID 8210, Figure 13) produced antibodies at substantially higher levels than those induced by two immunizations with the alum-precipitated toxoid. In addition, the monkeys boosted with the microsphere formulation by either mucosal route exhibited circulating IgA anti-toxin responses which were of a long duration. A point worthy of mention in the consideration of the levels of circulating IgA anti-toxin induced through the mucosal boosting of IM-primed monkeys, is the level of plasma IgG anti-toxin antibodies that are present in the plasma samples. The exceptionally high IgG antibody levels (titers of 819,200 to 1,638,400) must successfully compete for the solid-phase antigen in the assays, masking the true IgA anti-toxin level. Thus, the plasma IgA responses and the IgM responses, in these monkeys must be considered a minimum estimate.

(1) Oral boosting of IM-primed Monkey ID 8001

No increase in the level of circulating anti-toxin antibodies was observed following oral administration of 1 mg of microencapsulated TTD toxoid to one of the IM-primed monkeys (ID 8001, Figure 11). The Day 49 IgG anti-toxin titer of 409,600 fell to 51,200 by Day 70 and then remained at that level through Day 91. It is difficult to determine from these data if the plateau in circulating IgG antibodies observed on Day 70 through 91 represents maintenance through effective oral boosting or normal kinetics of the antibody decay after the primary IM immunization. However, this monkey, receiving the IM-oral immunizations, and Monkey ID 8161 (IM-IT), were the only monkeys to maintain a detectable plasma IgA anti-toxin level throughout the secondary response.

(2) IT boosting of IM-primed Monkey ID 8161

IT boosting of previously IM-immunized Monkey ID 8161 with 100 mg of microencapsulated TTD toxoid stimulated a prompt and vigorous increase in the circulating IgG anti-toxin antibody level. As shown in Figure 12, a titer of 1,638,400 was achieved on Day 63. This high titer was maintained through the time which the secondary responses were followed, and had only decreased to a titer of 819,200 on Day 98. As in the case of the monkey administered the IM-oral immunization schedule, this monkey exhibited circulating IgA anti-toxin antibodies through Day 98 when the analysis was terminated.

The exceptionally good boosting obtained by the IT route in this monkey, and in the other two monkeys which were boosted by the IT route (ID 8001, Figure 13 and ID 8044, Figure 14), indicate that the change in the method of IT installation, discussed previously, has significantly improved the effectiveness of this immunization route.

(3) IM boosting of IM-primed Monkey ID 8210

IM boosting of previously IM-immunized Monkey ID 8210 stimulated approximately a 16-fold rise in the plasma IgG anti-toxin level from a titer of 102,400 on Day 49 when the boost was administered, to a titer of 1,638,400 on Day 63 (Figure 13). A plateau titer of 819,200 was present on Days 70 through 98. In contrast to the IM-immunized monkeys which received mucosal booster immunizations, this monkey did not exhibit a detectable plasma IgA anti-toxin response after boosting

b. Secondary immunizations of IT-primed monkeys

For the reasons discussed previously, the monkeys receiving a primary IT immunization with the microencapsulated SEB toxoid were poorly primed. However, both the IT and IM routes proved to be effective boosters in these monkeys. The levels of IgG anti-toxin antibodies produced by these secondary immunizations were greater than those obtained in 2 of the 3 monkeys receiving a primary IM immunizations.

(1) Oral boosting of IT-primed Monkey 89 B072

Oral boosting of this IT-primed monkey did not result in the induction of a secondary circulating anti-toxin response that was detectable in any isotype, at any time point tested (Figure 14). However, there is evidence that the oral boosting did stimulate the appearance of specific anti-toxin antibodies at low level in the BAW fluid samples of this monkey.

(2) IT boosting of IT-primed Monkey 89 B001

IT boosting of Monkey 89 B001, which had received a primary IT immunization, induced the appearance and a rapid rise in the level of plasma IgG antibodies (Figure 15). On experimental Day 70, these antibodies reached a titer of 204,800, and this level was maintained through Day 98.

(3) IM boosting of IT-primed Monkey 89 B116

Following an IM boost with SEB toxoid microspheres, IT-primed Monkey 89 B116 exhibited a rapid rise in both IgM and IgG anti-toxin antibodies in the circulation (Figure 16). With the exception of the IgM component, the response by this monkey to the IT-IM regimen was quite similar to that seen in the monkey administered the IT-IT schedule. The IgG anti-toxin titer was 204,800 on Day 98 of the study.

c. Secondary immunizations of orally-primed monkeys

The monkeys administered a primary oral immunization with the microencapsulated SEB toxoid were clearly responsive to secondary immunization via systemic (IM) and mucosal (IT) routes. The 204,800 titer of circulating IgG anti-toxin achieved by both IT (Monkey 89 B064) and IM (Monkey 89 B034) boosting clearly shows that systemic tolerance has not been induced through oral immunization with the microencapsulated toxoid. That both these monkeys achieved circulating titers greater than those induced in 2 of the 3 monkeys receiving primary IM immunization with the microencapsulated SEB toxoid suggests that a degree of systemic priming has taken place. Somewhat puzzling is the total lack of a response in Monkey 89 B060 to both primary and secondary oral immunization with the SEB toxoid microspheres, when two other monkeys made clear responses to a single oral immunization. Taken together with the fact that Monkey 89 B079 was clearly nonresponsive to oral boosting, these data suggest a threshold effect in which only some of the monkeys are receiving an effective amount of vaccine. To what extent genetic factors may play a role is not known.

(1) Oral boosting of orally-primed Monkey 89 B060

Monkey 89 B060 failed to produce a detectable plasma response following either the primary or secondary oral immunization with microencapsulated SEB toxoid (Figure 17).

(2) IT boosting of orally-primed Monkey 89 B064

IT boosting of Monkey 89 B064, which had made the greatest plasma response to a primary oral immunization, resulted in an immediate rise in the levels of IgG anti-toxin antibodies. This response reached a titer of 204,800 on Day 56 (Figure 18). The titer remained steady at this level through Day 98. This indicates that the modification to the IT immunization procedure provided an effective immunization with the microencapsulated SEB toxoid.

(3) IM boosting of orally-primed Monkey 89 B034

This monkey, which had also responded to the primary oral immunization, produced a clear response to secondary IM immunization (Figure 19). Unlike the orally-primed monkey that was boosted by the IT route, this monkey's titer of IgG anti-toxin rose progressively through Day 77 to a titer of 204,800. On Experimental Days 77 through 98, the plasma IgG anti-toxin level remained steady.

3. BAW anti-SEB toxin responses from EX-Rh-101

At no time did any of the samples of BAW fluids from the control, nonimmunized monkey (89 B088) contain detectable anti-SEB toxin antibodies. These results are presented in Table 5, which contains all of the BAW fluids analysis for EX-Rh-101.

The BAW fluids from the monkeys which received a primary IM immunizations with microencapsulated SEB toxoid (89 B096, 89 B161 and 89 B210) were without anti-toxin activity except for the Day 49 sample from Monkey 89 B096. This sample contained specific anti-SEB toxin IgG antibodies at a titer of 80. In contrast, 2 of the 3 monkeys administered a primary immunization by the IT route (89 B079, 89 B001 and 89 B116) exhibited IgM anti-toxin antibodies in their BAW fluids samples at a titer of 40. (Note: These are the monkeys which had received the primary IT immunization as a bolus deposition of the SEB toxoid microspheres deep into one lung lobe. The method of IT immunization was altered for later immunizations. The monkeys must be considered poorly primed by this immunization). The monkeys administered a primary oral immunization (89 B060, 89 B064 and 89 B034) did not mount a primary immune response which was detectable in their BAW fluids, in any isotype, at the time points tested.

Booster immunization of the IM-primed monkeys, regardless of the route of administration, resulted in enhanced levels of IgG anti-toxin antibodies in the BAW fluids. However, only the monkey boosted via the IT route showed a detectable IgA anti-toxin response.

Booster immunization of the IT-primed monkeys by the IT route (89 B001) and the IM route (89 B116) resulted in the appearance of IgG anti-toxin antibodies in the BAW fluids, while the BAW fluids from the orally-boosted monkey continued to contain IgM.

The orally-primed monkeys which were boosted by the oral (89 B060) and IM (89 B034) routes failed to produce detectable responses in their BAW fluids. However, IT boosting of orally-primed Monkey 89 B064, resulted in both IgG and IgA anti-toxin antibodies that were still detectable on Day 98. The antibody titers were 80 and 20 for the IgG and IgA respectively.

D. Immunization Results of Monkey Study EX-Rh-102

The immunization schedule for the monkeys in this experiment is the same as that for the previous study. This schedule is summarized in Table 6.

1. Plasma anti-SEB toxin responses following primary immunizations

The nonimmunized, control monkey (90 B001) did not exhibit detectable plasma anti-SEB toxin antibodies of any isotype, at any time point tested. This is illustrated in Figure 20.

a. Primary IM immunization (Monkeys 90 B002, 90 B003 and 90 B010)

Each of the monkeys which were administered a primary immunization with 100 μg of microencapsulated SEB toxoid mounted a brisk circulating anti-toxin antibody response. This response was primarily in the IgG isotype, but also in the IgM isotype. These immune responses are graphically depicted in the indicated figures:

Monkey	Figure
90 B002	21
90 B003	22
90 B010	23

The responses by all the monkeys receiving a primary IM immunization with the SEB toxoid microspheres were similar in that their maximal primary IgG anti-toxin titers of 51,200 to 102,400 were attained on Day 21 and remained at this level through Day 49 when booster immunizations were administered. Thus, as in Study EX-Rh-101, each of these monkeys attained primary IgG anti-toxin levels which equalled or exceeded the highest peak primary IgG anti-toxin titer achieved by a monkey immunized with an equal dose of SEB toxoid precipitated on alum. In addition, the monkeys immunized with the microencapsulated toxoid exhibited no decline in their Day 21 antibody titers.

b. Primary IT immunization (Monkeys 90 B023, 90 B009 and 90 B021)

It should be noted that the altered procedure for IT immunization was used for these primary immunizations. Microspheres containing 100 μg of SEB toxoid, administered via the IT route, resulted in circulating anti-SEB toxin responses in all three monkeys. The plasma IgG titers (102,400 to 409,600) equalled or exceeded the responses induced by IM immunization with alum-precipitated or microencapsulated SEB toxoid. Figures showing the immune responses for these monkeys are as follows:

Monkey	Figure
90 B023	24
90 B009	25
90 B021	26

In addition to the strong IgG anti-toxin response induced via IT immunization with the microencapsulated SEB toxoid, this approach also induced circulating IgM responses which tended to peak early and fall to undetectable levels by Day 35. IgA anti-toxin antibodies were also detectable through Day 49 when the booster immunizations were administered.

c. Primary oral immunization (Monkeys 90 B014, 90 B008 and 90 B018)

Following oral immunization with SEB toxoid microspheres, all three of the monkeys produced circulating anti-SEB toxin antibodies. There were some differences in the responses, which are illustrated in the indicated figures.

Monkey	Figure
90 B014	27
90 B008	28
90 B018	29

Monkeys 90 B014 and 90 B018 produced IgM anti-toxin antibodies on Day 14 followed by the appearance of IgG antibodies on Day 21. However, Monkey 90 B014 exhibited these circulating IgG antibodies through Day 49, while the IgG response in Monkey 90 B018 declined to undetectable levels on Day 42. In contrast, Monkey 90 B008 produced circulating IgM and IgA anti-toxin antibodies that were only detected on Day 35. Although none of these monkeys mounted high titers of circulating anti-toxin antibodies, when the results from this experiments are combined with those from Study EX-Rh-101, 5 of 6 monkeys receiving the microencapsulated SEB toxoid orally, have produced circulating antibody responses. This is in contrast to Study EX-Rh-100, in which neither of the two monkeys administered non-microencapsulated SEB toxoid exhibited any detectable response.

2. Plasma anti-SEB toxin responses following secondary immunizations

a. Secondary immunizations of IM-primed monkeys

As a group, the monkeys administered primary IM immunizations with microencapsulated SEB toxoid, and boosted either systemically or by the two mucosal routes, exhibited good levels of circulating anti-toxin antibodies. The orally-boosted monkey, 90 B002 (Figure 21), maintained an IgG antibody level which was of the same magnitude as monkeys administered primary and secondary immunizations with SEB toxoid precipitated on alum. The IT-boosted monkey, 90 B003 (Figure 22) and the IM-boosted monkey, 90 B010 (Figure 23) produced antibodies at substantially higher levels than those induced by two immunizations with the alum precipitated toxoid.

In addition, the monkey boosted with the SEB toxoid microspheres by the IT route, exhibited circulating IgA anti-toxin responses which were of a long duration. A point worthy of mention in the consideration of the levels of circulating IgA anti-toxin induced through the mucosal boosting of IM-primed monkeys, is the level of plasma IgG anti-toxin which is present in the plasma samples. The exceptionally high IgG antibody levels (titers of 819,200 to 1,638,400) must successfully compete for the solid-phase antigen in the assays, masking the true IgA anti-toxin level. Thus, the plasma IgA and IgM responses in these monkeys must be considered minimum estimates.

(1) Oral boosting of IM-primed Monkey 90 B002

Following the oral administration of 1.0 mg of microencapsulated SEB toxoid to this IM-primed monkey, a booster response was evident in the IgM isotype on Days 56 through 70 (Figure 21). Although the circulating IgG anti-toxin titer fell from its Day 63 high, it remained on a plateau from Day 77 through Day 98. This suggests that the oral booster may have helped maintain the circulating antibody level.

(2) IT boosting of IM-primed Monkey 90 B003

An IT boost consisting of 100 μg of microencapsulated SEB toxoid resulted in a prompt and vigorous response in this monkey. As shown in Figure 22, the circulating IgG anti-toxin antibody level increased to a titer of 1,638,400 on Day 63. This high titer of IgG anti-toxin activity was maintained through the time which the secondary response was followed. The response had only decreased to a titer of 819,200 on Day 98. In addition, this monkey exhibited circulating IgA anti-toxin antibodies ten days after IT boosting. These IgA antibodies rose steadily to a titer of 1,600 on Day 77 and remained at this level though Day 98.

(3) IM boosting of IM-primed Monkey 90 B010

IM boosting of previously IM-immunized Monkey 90 B010 stimulated approximately a 16-fold rise in the plasma IgG anti-toxin level. As illustrated in Figure 23, the IgG anti-toxin antibody titers increased from 102,400 on Day 49 to 1,638,400 on Day 63. A plateau titer of 409,600 was present on Days 77 through 98. In contrast to the IM-immunized monkeys which received mucosal booster immunizations, this monkey did not exhibit a detectable IgA anti-toxin response following the booster immunization.

b. Secondary immunizations of IT-primed monkeys

The monkeys which received IT primary immunizations with microencapsulated SEB toxoid were very well primed. This demonstrated that the change in the IT installation procedure has been effective. Both the IT and IM routes proved to be very effective boosters in these monkeys. The long-term high levels of IgG antibodies in the monkeys receiving an oral booster suggests that an effective amount of vaccine was delivered by this route.

(1) Oral boosting of IT-primed Monkey 90 B023

Oral boosting of this IT-primed monkey did not result in the induction of a clearly demonstrable booster response (Figure 24). However, good maintenance of the circulating IgG anti-toxin levels are consistent with the delivery of an effective amount of vaccine.

(2) IT boosting of IT-primed Monkey 90 B009

Following the IT boost, a rapid rise in the levels of plasma IgG and IgA anti-toxin antibodies was detectable (Figure 25). The IgG antibodies reached a titer of 819,200 on Day 63 and remained at this level through the duration of the experiment. The IgA antibodies reached a peak titer of 6,400 at a point 10 days after the IT boost, and were present at elevated titers throughout the experiment.

(3) IM boosting of IT-primed Monkey 90 B021

After an IM boost with SEB toxoid microspheres, this IT-primed monkey exhibited a rapid rise in IgG anti-toxin antibodies in the circulation (Figure 26). The secondary response by this monkey was very similar to that seen in the monkey receiving two IM immunizations. The IgG anti-toxin titer rose to 1,638,400 on Day 63 and then remained at a plateau level of 204,800 through Day 98. However, this secondary response differs significantly from that exhibited by the IT-IT immunized monkey in that it completely lacks a detectable IgA response.

c. Secondary immunizations of orally-primed monkeys

Each of the three monkeys administered a primary oral immunization responded with circulating anti-toxin antibodies. In addition, all were clearly responsive to secondary immunization via the systemic and mucosal routes. The titers of circulating IgG anti-toxin achieved by both IT (90 B008) and IM (90 B018) boosted monkeys indicated that systemic tolerance had not been induced through oral immunization with the microencapsulated SEB toxoid.

(1) Oral boosting of orally-primed Monkey 90 B014

In the first 21 days following oral boosting of the orally-primed monkey, a four-fold rise in the circulating IgG anti-toxin antibodies to a titer of 800 was observed (Figure 27). Once attained, this titer remained constant through Day 98. However, this immunization method did not result in the appearance of detectable IgA anti-toxin activity at any tested time point.

(2) IT boosting of orally-primed Monkey 90 B008

IT boosting of this monkey resulted in a rise in the levels of IgG anti-toxin antibodies which was evident 14 days after boosting. The increase continued through Experimental Day 77, when the titer reached 102,400. Thereafter the titer decreased to 25,600 on Day 98 (Figure 28).

(3) IM boosting of orally-primed Monkey 90 B018

This monkey exhibited a rapid response to a secondary IM immunization which was characterized by both IgM and IgG components (Figure 29). The IgM response was of short duration and had fallen to an undetectable level 28 days after boosting. In contrast, the IgG anti-toxin response steadily increased through Day 77 to a titer of 25,600 and remained at that level through Day 98.

3. BAW anti-SEB toxin responses from EX-Rh-102

As in Study EX-Rh-101, the nonimmunized control monkey in this experiment did not exhibit detectable anti-toxin antibodies of any isotype in any of the samples tested. Table 7 contains this data as well as BAW data from all the monkeys in Study EX-Rh-102.

None of the monkeys administered a primary IM (90 B002, 90 B003 and 90 B010) or primary oral (90 B014, 90 B008 and 90 B018) immunization with the microencapsulated SEB toxoid produced an anti-toxin response that was detectable in the BAW fluids. In contrast, all the monkeys receiving a primary immunization via the IT route, responded with relatively high levels of IgA and IgG anti-toxin antibodies in their BAW fluids.

Booster immunization of the IM-primed monkeys by the oral route (90 B002) and IM route (90 B010) was ineffective at stimulating an anti-toxin response in the BAW fluids. However, boosting by the IT route (90 B003) induced appearance of IgG and IgA anti-toxin antibodies at titers of 12,800 and 320, respectively.

Each of the monkeys primed by the IT route continued to have measurable anti-toxin antibodies after boosting. The antibody levels declined, despite the boost in the orally-boosted monkey (90 B023). In the IT-boosted monkey (90 B009), the IgA antibodies in BAW fluids increased and the IgG antibodies decrease after boosting. The converse was evident for the IM-boosted monkey (90 B021).

None of the orally-primed monkeys (90 B014, 90 B008 and 90 B018) mounted a detectable response in the BAW fluids regardless of the route of booster immunization.

VII. DISCUSSIONS AND CONCLUSIONS

During this year of the contract, we obtained a great deal of knowledge about the immunization of rhesus monkeys with SEB toxoid. A preliminary study was performed to confirm the existing data regarding the effectiveness of systemic (IM) immunization with alum precipitated SEB toxoid and oral immunization with high doses of toxoid in solution, to raise circulating anti-SEB toxin antibodies. Additionally, several studies involving the immunization of rhesus monkeys with SEB toxoid microspheres have also been performed.

The preliminary monkey experiment yielded the following results: Immunization with alum-precipitated SEB toxoid did result in the appearance of circulating IgG antibodies, which were detected in low levels in the BAW fluids. Oral immunization with SEB toxoid solution induced only the transient appearance of low levels of IgM and IgA anti-SEB toxin antibodies in circulation.

Results from the first two studies (EX-Rh-101 and EX-Rh-102) in which rhesus monkeys were immunized with microencapsulated SEB toxoid are included in this report. These data suggest that an IM primary immunization followed by an IT or oral secondary immunization provides the highest antibody response and best level of protection. It has been reported to us that the monkeys receiving an IM primary and IT secondary immunization in both studies survived aerosol challenge. In addition, the monkey receiving an oral primary and IT secondary immunization from Study EX-Rh-101 and the monkey receiving both an IT primary and IT secondary immunization in Study EX-Rh-102 also survived the aerosol challenge. Official documentation of these results has not been received by Southern Research or the University of Alabama in Birmingham.

These results are very encouraging as not only have high antibody titers been achieved in the rhesus monkeys, but as indicated above, several of the monkeys have been shown to be protected from aerosol challenge.

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IX. ACKNOWLEDGMENTS

Ms. Cheryl D. Hudson, Research Chemical Technician, prepared and characterized the SBR toroid microspheres. Mr. Orlan S. Finch, Research Assistant, is responsible for maintaining the mouse-breeding colony, animal handling, tissue procurement, and reagent preparation. Ms. Charlotte K. Jones, Research Assistant II, is responsible for tissue sampling, histological sectioning, immunohistochemical procedures, and monoclonal antibody production and purification.

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APPENDIX A

TABLES

TABLE 1. SEB MICROSPHERES PREPARED DURING THIS REPORTING PERIOD

Batch[a]	Batch size, g	Yield, %	Core loading, wt % SEB		Encapsulation efficiency, % of theoretical
			Theoretical	Actual	
F787-046-00	1.01	68.8	1.0	0.30	30.0
F787-052-00	1.01	62.0	1.0	0.35	35.0
F787-055-00	2.02	80.6	1.0	0.42	42.0
F787-071-00	2.02	77.3	1.0	--[b]	--
F787-073-00	2.02	79.3	1.0	--	--
F787-074-00[c]	--		1.0	0.48	48.0
F787-082-00	2.02	81.2	1.0	--	--
F787-089-00	2.02	75.9	1.0	--	--
F787-091-00	2.02	77.1	1.0	--	--
F787-093-00	2.02	79.9	1.0	--	--
F787-095-00	2.02	78.7	1.0	--	--
F787-110-00[d]	--	--	1.0	0.41	41.0
F787-117-00	2.02	--	1.0	--	--
F787-119-00	2.02	--	1.0	--	--
F787-121-00	2.02	--	1.0	--	--
F787-123-00	2.02	--	1.0	--	--
F787-124-00[e]	--	78.0	1.0	0.35	35.0

[a] All microspheres were prepared with a 53:47 DL-PLG excipient. Lot BPI-043-62-1.

[b] "--" = Not determined.

[c] Batch F787-074-00 is a composite of Batches F787-071-00 and F787-073-00.

[d] Batch F787-110-00 is a composite of Batches F787-082-00, -089-00, -091-00, -093-C0, and -095-00.

[e] Batch F787-124-00 is a composite of Batches F787-117-00, -119-00, -121-00, and -123-00.

TABLE 2. SIZE-DISTRIBUTION DATA FOR SEB MICROSPHERES PREPARED DURING THIS REPORTING PERIOD

Batch	By volume, % of microspheres		
	Less than 4.6 μm in diameter	From 4.6 to 9.6 μm in diameter	Greater than 9.6 μm in diameter
F787-046-00	95.7	4.3	0.0
F787-052-00	63.2	33.8	3.0
F787-055-00	87.2	12.8	0.0
F787-074-00	91.7	8.3	0.0
F787-082-00	53.5	40.0	6.5
F787-110-00	63.7	35.7	0.6
F787-124-00	73.2	25.5	1.3

TABLE 3. BAW ANTI-SEB TOXIN RESPONSE TO SEB TOXOID, EX-RH-100

Monkey number	Immunization schedule	Bronchial-alveolar wash anti-SEB toxin titer[a]											
		Day 28			Day 49			Day 77			Day 98		
		IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA
89 B109	Control[b]	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
89 B093	Control	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
89 B106	IM-IM[c]	<10	<10	<10	<10	40	<10	<10	80	<10	<10	10	<10
89 B115	IM-IM	<10	<10	<10	<10	20	<10	<10	80	<10	<10	10	<10
89 B043	O-O[d]	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
89 B103	O-O	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

[a] Anti-toxin titer determined by end-point titration.

[b] Not-immunized.

[c] Immunized on Days 0 and 49 by IM injection of 100 μg of SEB toxoid on alumn.

[d] Immunized on Days 0 and 49 by oral gavage of 10 mg of SEB toxoid in a 0.7% bicarbonate solution.

TABLE 4. SUMMARY OF IMMUNIZATIONS FOR STUDY EX-RH-101

Monkey number	Primary immunization Day 0		Secondary immunization Day 49	
	Route	Dose	Route	Dose
89 B088	None	None	None	None
89 B096	IM	100 μg	Oral	1 mg
89 B161	IM	100 μg	IT	100 μg
89 B210	IM	100 μg	IM	100 μg
89 B079	IT	100 μg	Oral	1 mg
89 B001	IT	100 μg	IT	100 μg
89 B116	IT	100 μg	IM	100 μg
89 B060	Oral	1 mg	Oral	1 mg
89 B064	Oral	1 mg	IT	100 μg
89 B034	Oral	1 mg	IM	100 μg

TABLE 5. BAW ANTI-SEB TOXIN RESPONSE TO SEB TOXOID MICROSPHERES, EX-RH-101

Monkey number	Immunization schedule	Bronchial-alveolar wash anti-SEB toxin titer[a]											
		Day 28			Day 49			Day 77			Day 98		
		IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA
89 B088	Control[b]	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
89 B096	IM-O	<10	<10	<10	<10	80	<10	20	1280	<10	<10	<10	<10
89 B161	IM-IT	<10	<10	<10	<10	<10	<10	<10	320	40	<10	320	40
89 B210	IM-IM	<10	<10	<10	<10	<10	<10	<10	80	<10	<10	40	<10
89 B079	IT-O	40	<10	<10	40	<10	<10	40	<10	<10	40	<10	<10
89 B001	IT-IT	<10	<10	<10	40	<10	<10	40	20	<10	40	<10	<10
89 B116	IT-IM	<10	<10	<10	<10	<10	<10	<10	20	<10	40	20	<10
89 B060	O-O	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
89 B064	O-IT	<10	<10	<10	<10	<10	<10	<10	320	40	<10	80	20
89 B034	O-IM	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

[a]Anti-toxin titer determined by end-point titration.

[b]Not-immunized.

TABLE 6. SUMMARY OF IMMUNIZATIONS FOR EXPERIMENT EX-RH-102

Monkey number	Primary immunization Day 0		Secondary immunization Day 49	
	Route	Dose	Route	Dose
90 B001	None	None	None	None
90 B002	IM	100 μg	Oral	1 mg
90 B003	IM	100 μg	IT	100 μg
90 B010	IM	100 μg	IM	100 μg
90 B023	IT	100 μg	Oral	1 mg
90 B009	IT	100 μg	IT	100 μg
90 B021	IT	100 μg	IM	100 μg
90 B014	Oral	1 mg	Oral	1 mg
90 B008	Oral	1 mg	IT	100 μg
90 B018	Oral	1 mg	IM	100 μg

TABLE 7. BAW ANTI-SEB TOXIN RESPONSE TO SEB TOXOID MICROSPHERES, EX-RH-102

Monkey number	Immunization schedule	Bronchial-alveolar wash anti-SEB toxin titer[a]											
		Day 28			Day 49			Day 77			Day 98		
		IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA
90 B001	Control[b]	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
90 B002	IM-O	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	20
90 B003	IM-IT	<10	<10	<10	<10	<10	<10	<10	320	160	<10	1280	320
90 B010	IM-IM	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
90 B023	IT-O	<10	1280	160	<10	320	20	<10	20	<10	<10	20	<10
90 B009	IT-IT	<10	640	40	<10	1280	40	<10	640	320	<10	320	160
90 B021	IT-IM	<10	160	80	<10	160	80	<10	320	20	<10	320	<10
90 B014	O-O	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
90 B008	O-IT	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
90 B018	O-IM	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

[a]Anti-toxin titer determined by end-point titration.

[b]Not-immunized.

APPENDIX B

FIGURES

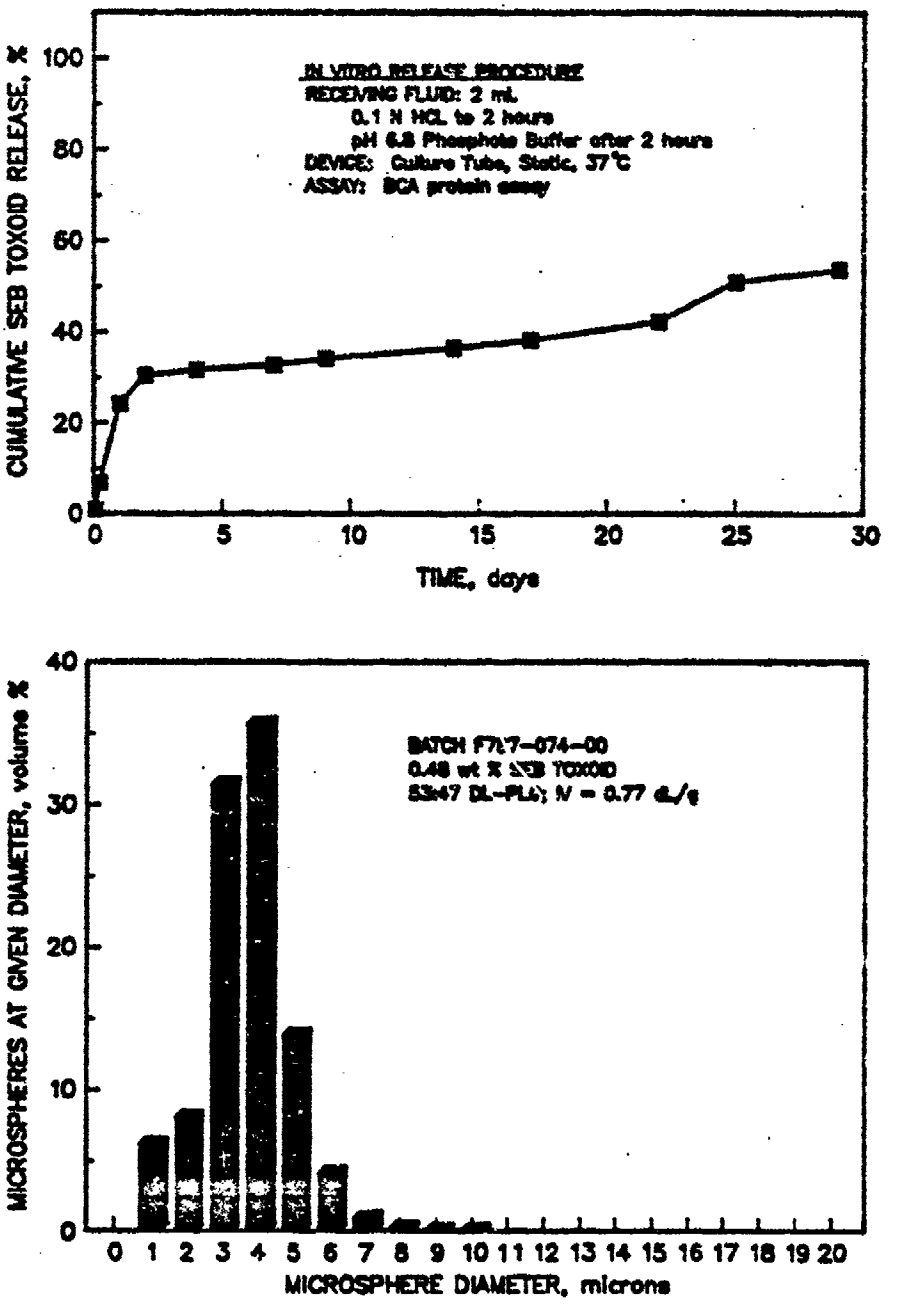
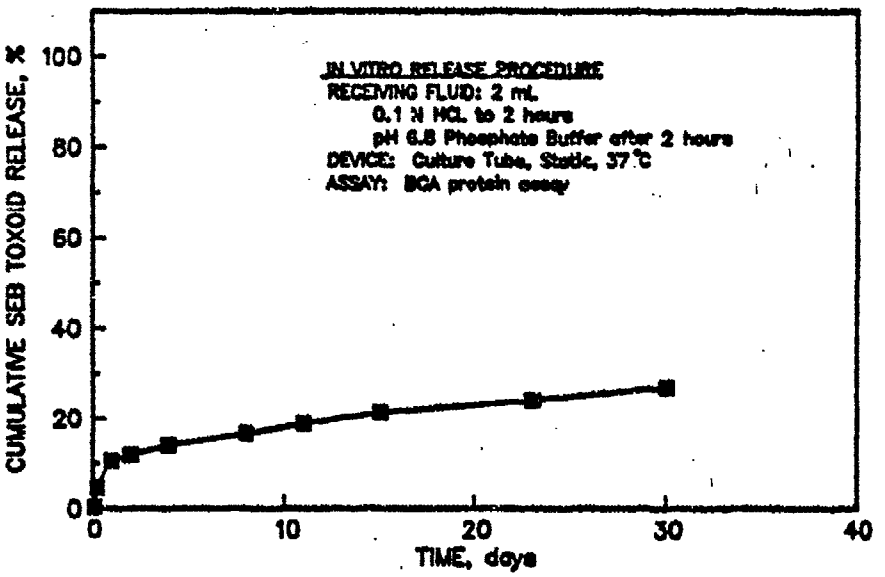


Figure 1. Cumulative in vitro release of SEB toxoid (above) and microsphere size distribution (below): Batch F787-074-00.



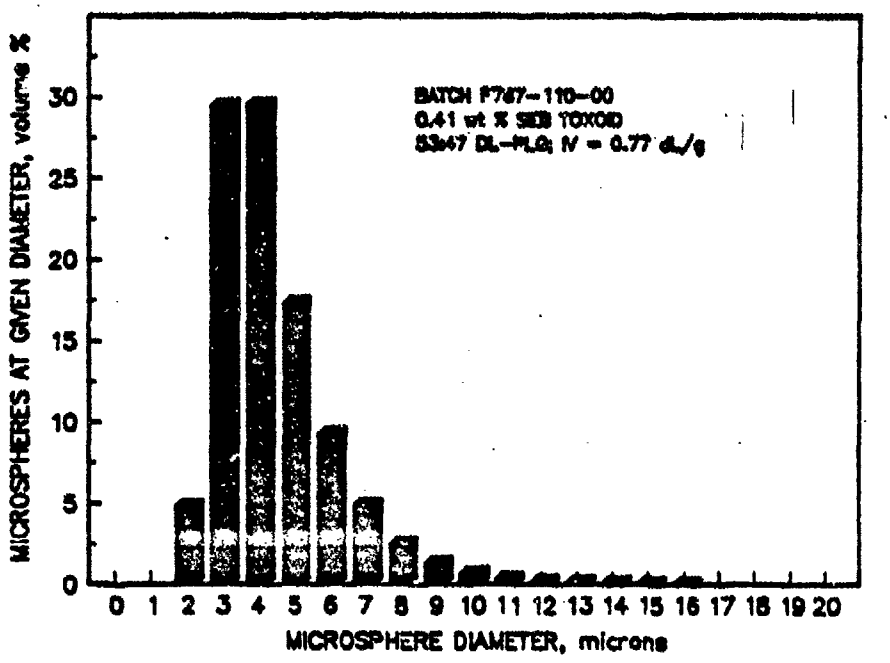


Figure 2. Cumulative in vitro release of SEB toxoid (above) and microsphere size distribution (below): Batch F787-110-00.

PROPRIETARY INFORMATION
(COMPLETE PAGE)

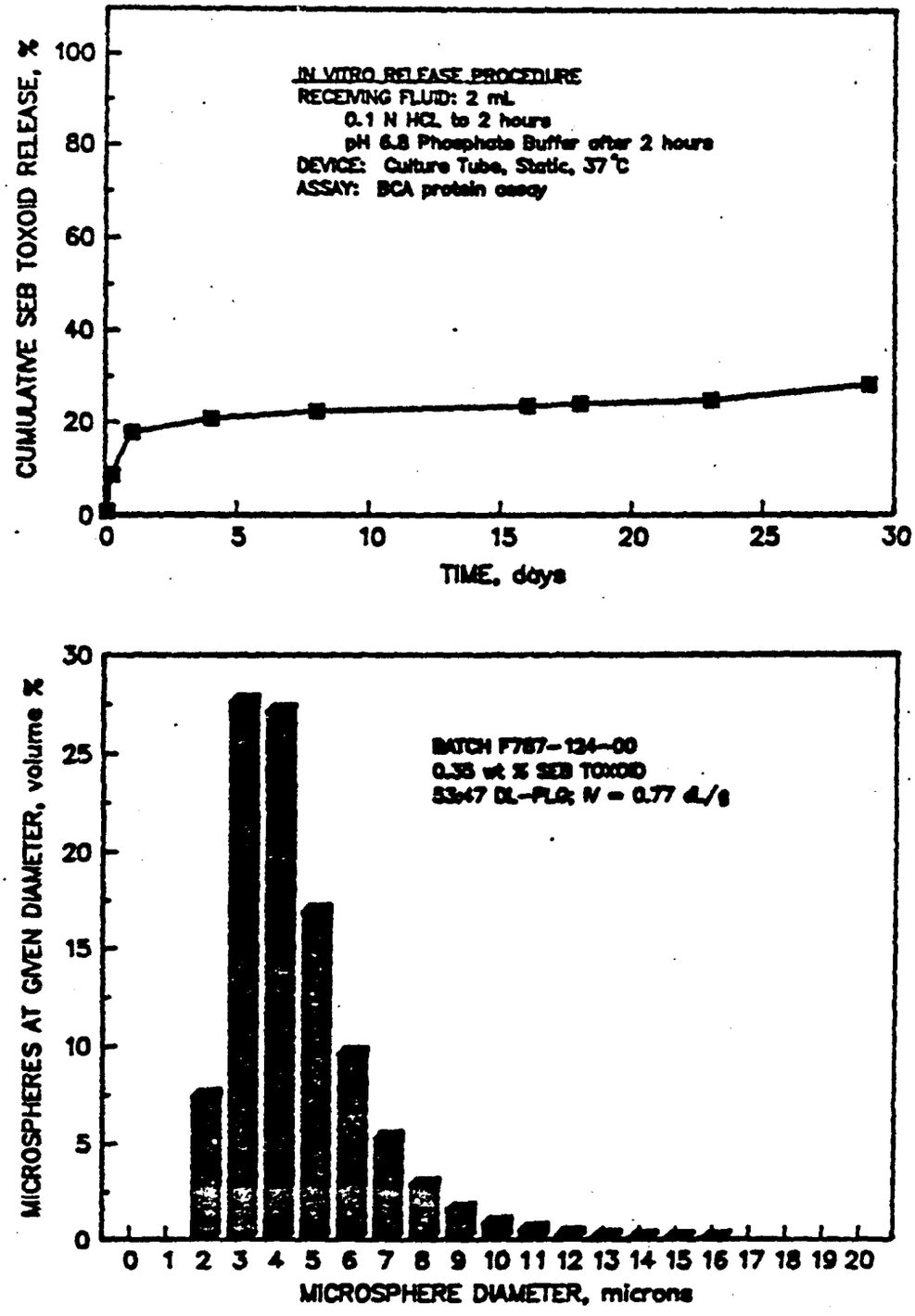


Figure 3. Cumulative in vitro release of SEB toxoid (above) and microsphere size distribution (below): Batch F787-124-00.

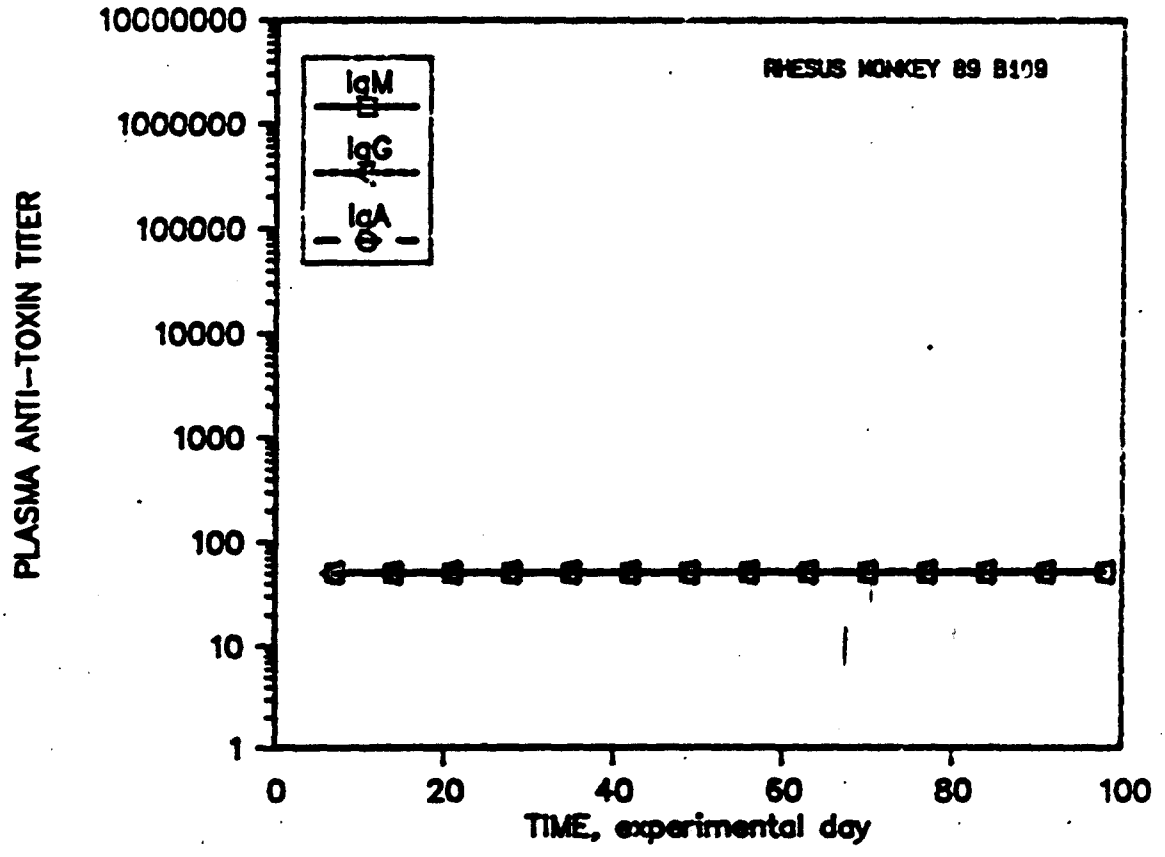


Figure 4. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 89 B109: Non-immunized control.

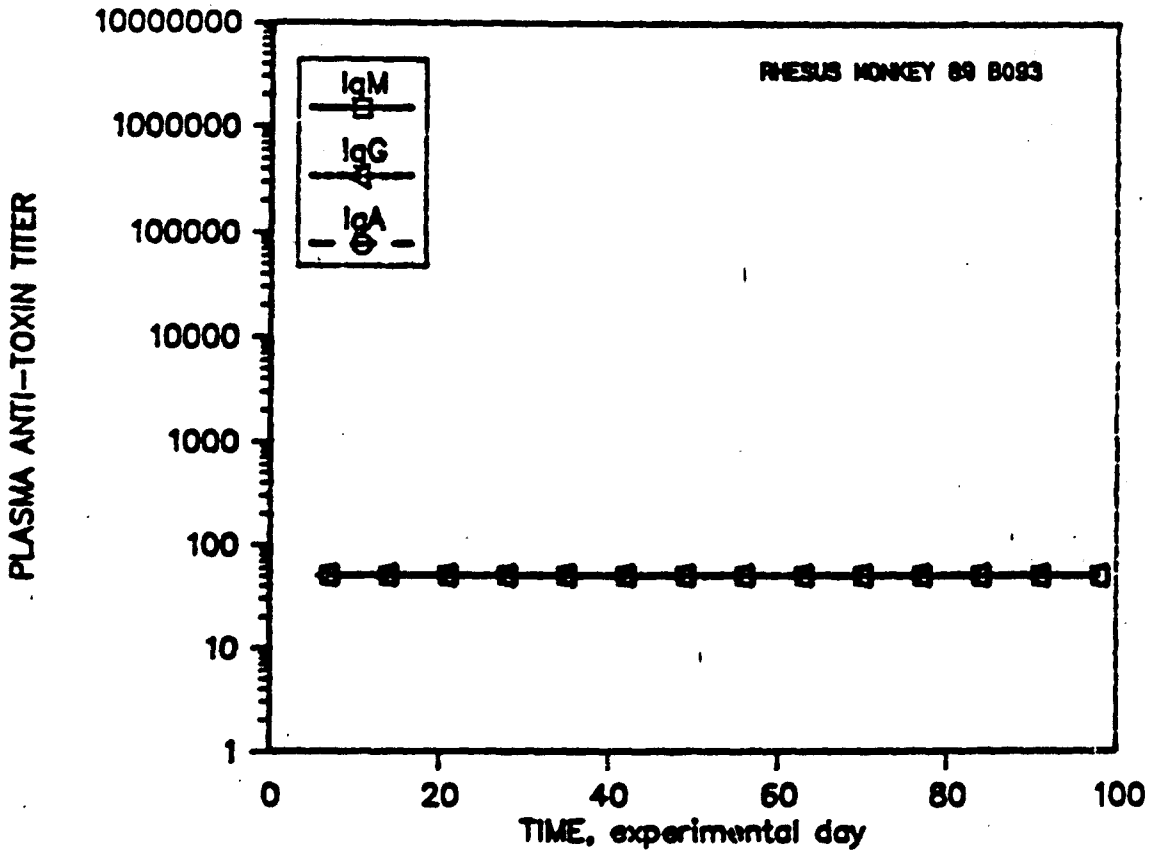


Figure 5. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 89 B093: Non-immunized control.

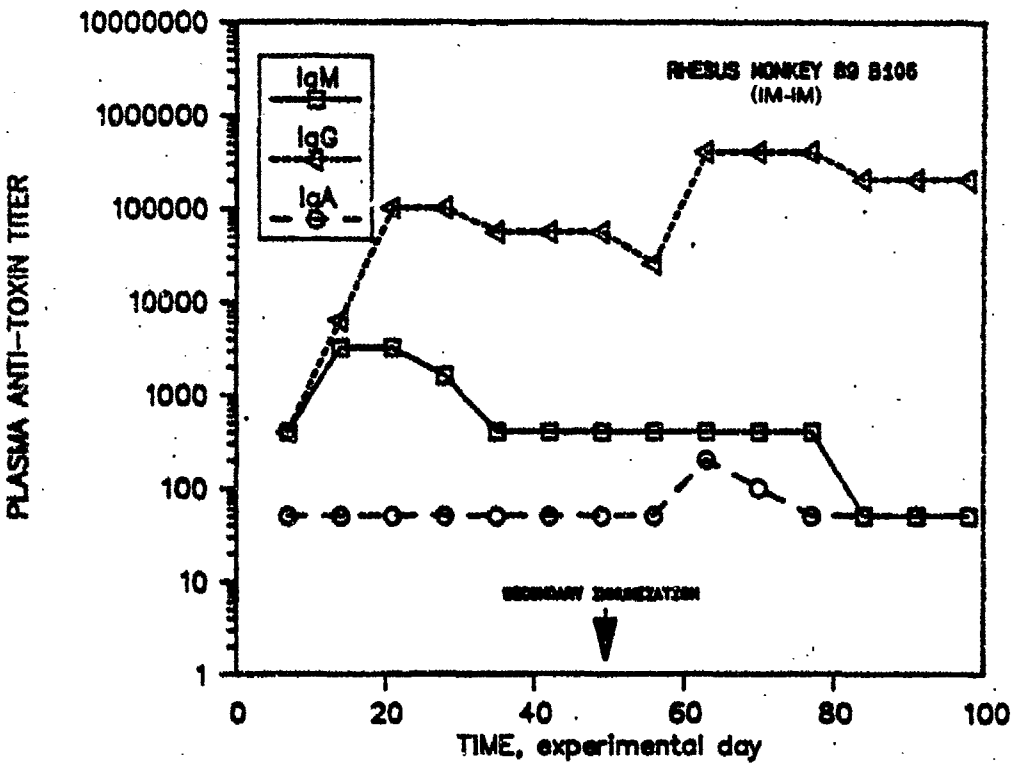


Figure 6. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 89 B106: Immunized by IM injection on Days 0 and 49 with 100 micrograms of SEB toxoid precipitated on alum.

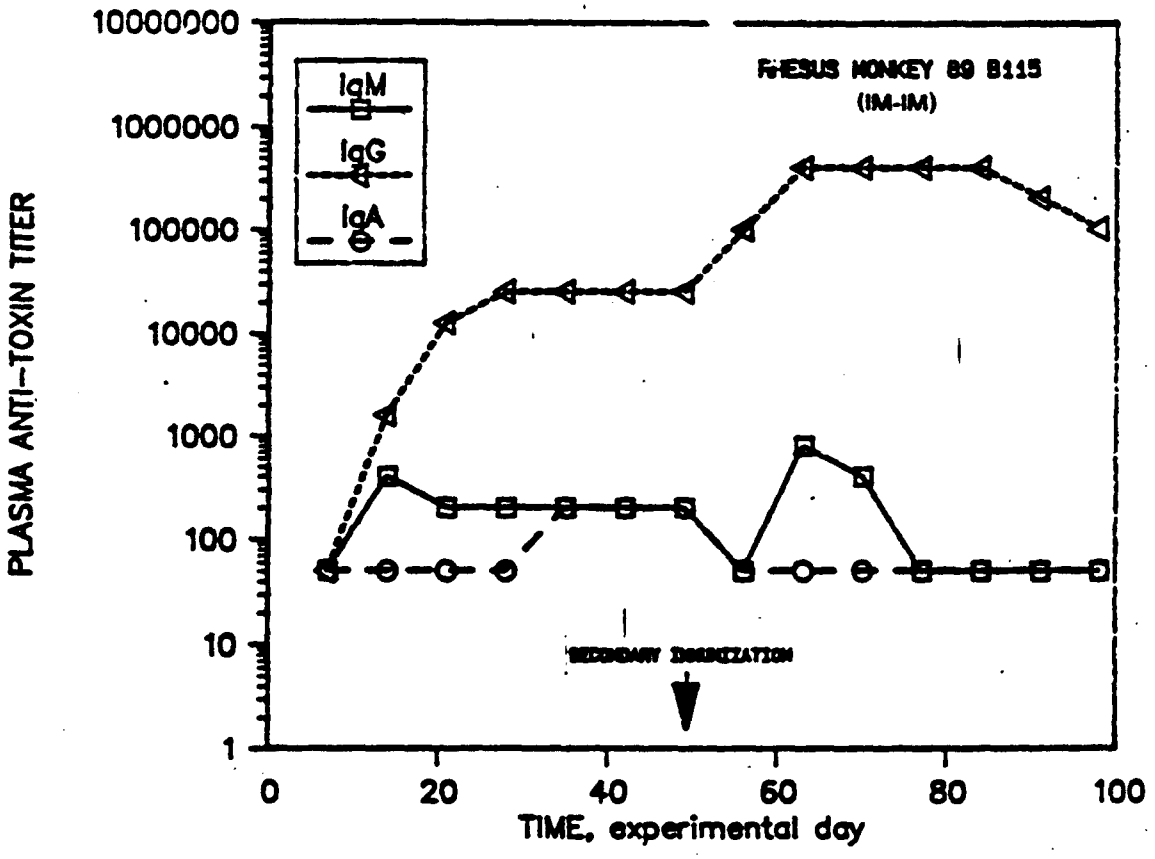


Figure 7. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 89 B115: Immunized by IM injection on Days 0 and 49 with 100 micrograms of SEB toxoid precipitated on alum.

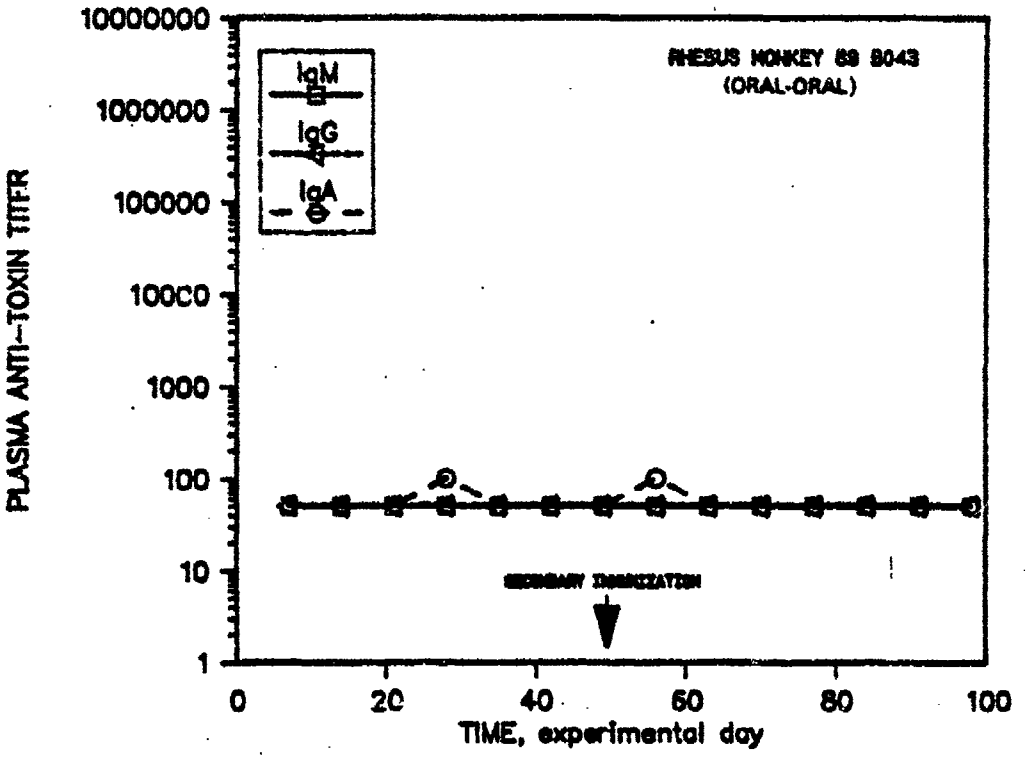


Figure 8. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 89 B043: Immunized by oral gavage, on Days 0 and 49 with 10 mg of SEB toxoid in 5.0 mL of 0.7% bicarbonate.

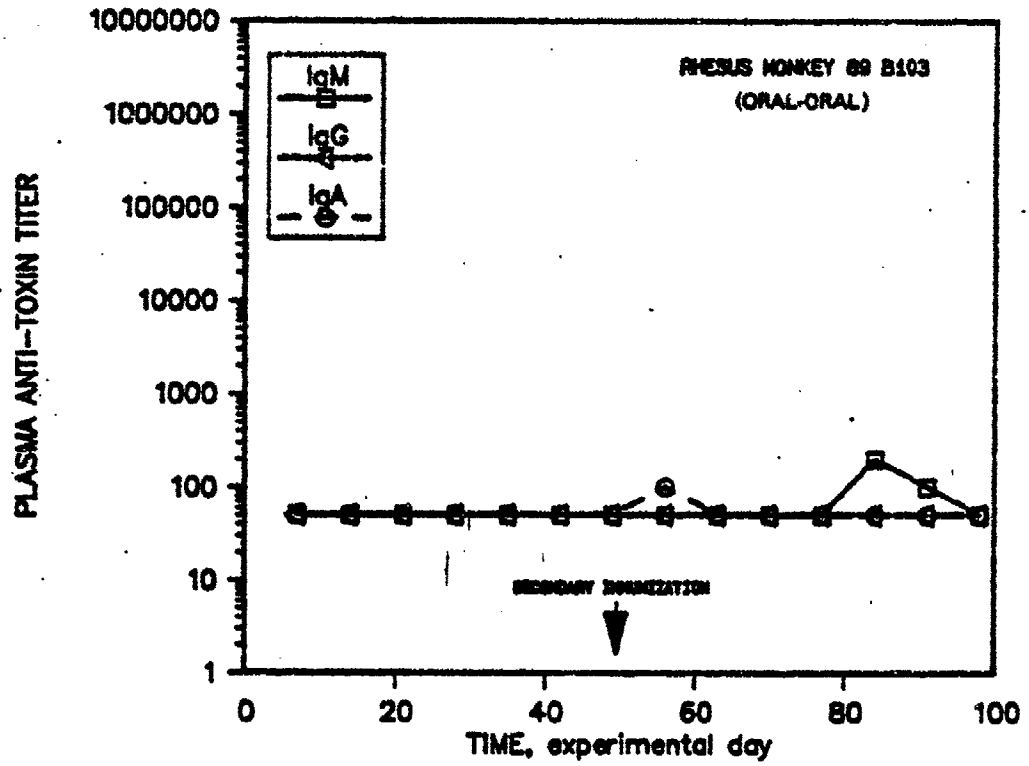


Figure 9. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 89 B103: Immunized by oral gavage on Days 0 and 49 with 10 mg of SEB toxoid in 5.0 mL of 0.7% bicarbonate.

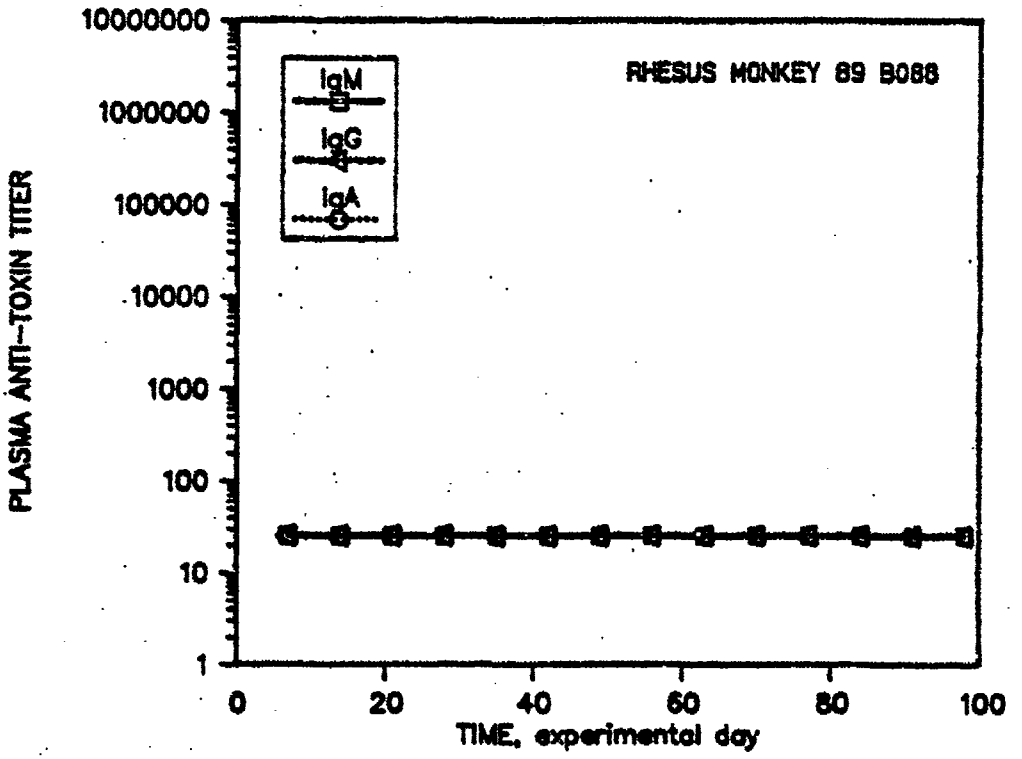


Figure 10. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 89 B088: Non-immunized control.

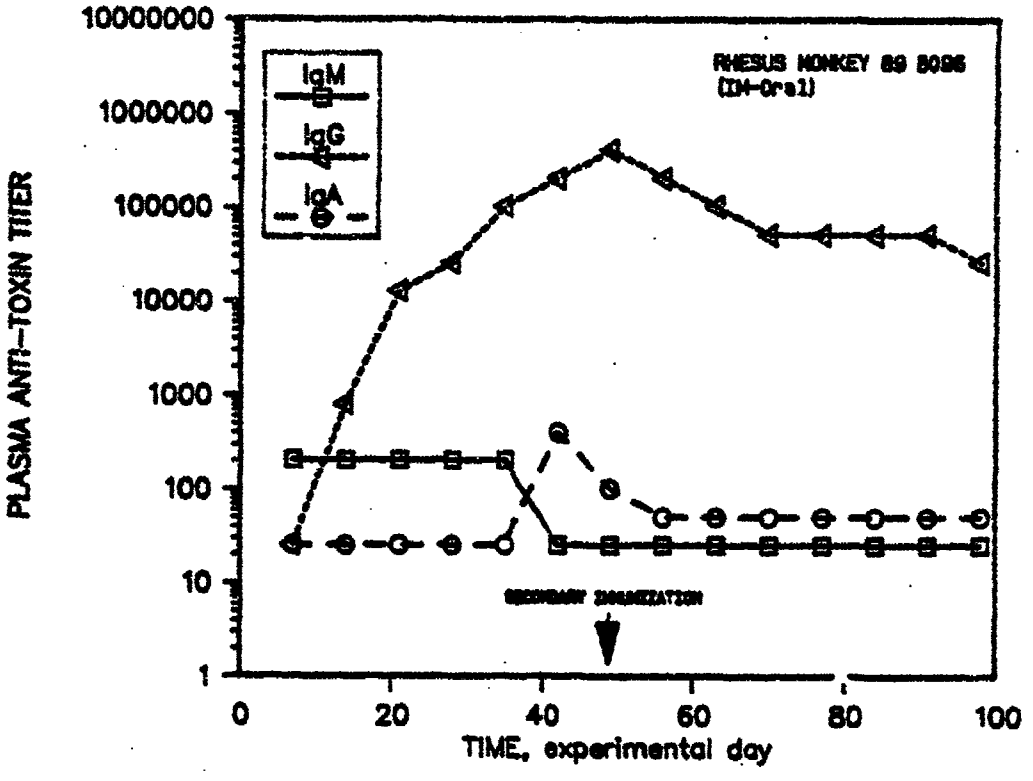


Figure 11. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 89 B096: Primary immunization (Day 0)—100 micrograms microencapsulated SEB toxoid by IM injection. Secondary immunization (Day 49)—1.0 mg of microencapsulated SEB toxoid by oral gavage.

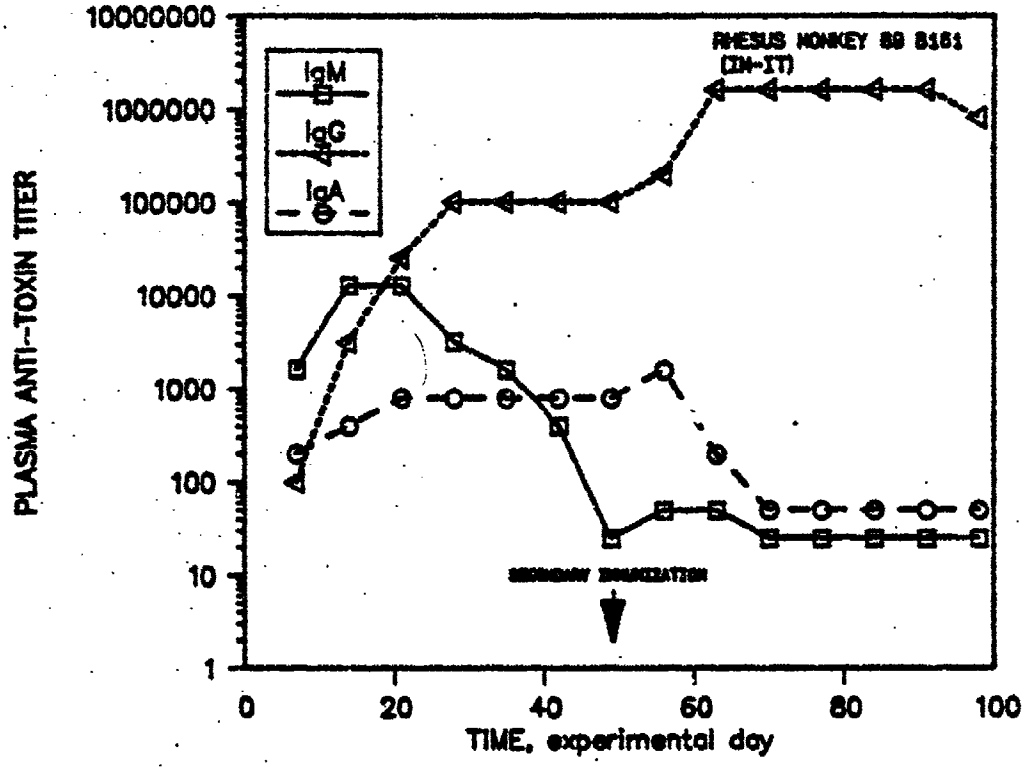


Figure 12. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 89 B161: Primary immunization (Day 0)—100 micrograms microencapsulated SEB toxoid by IM injection. Secondary immunization (Day 49)—100 micrograms of microencapsulated SEB toxoid by IT instillation.

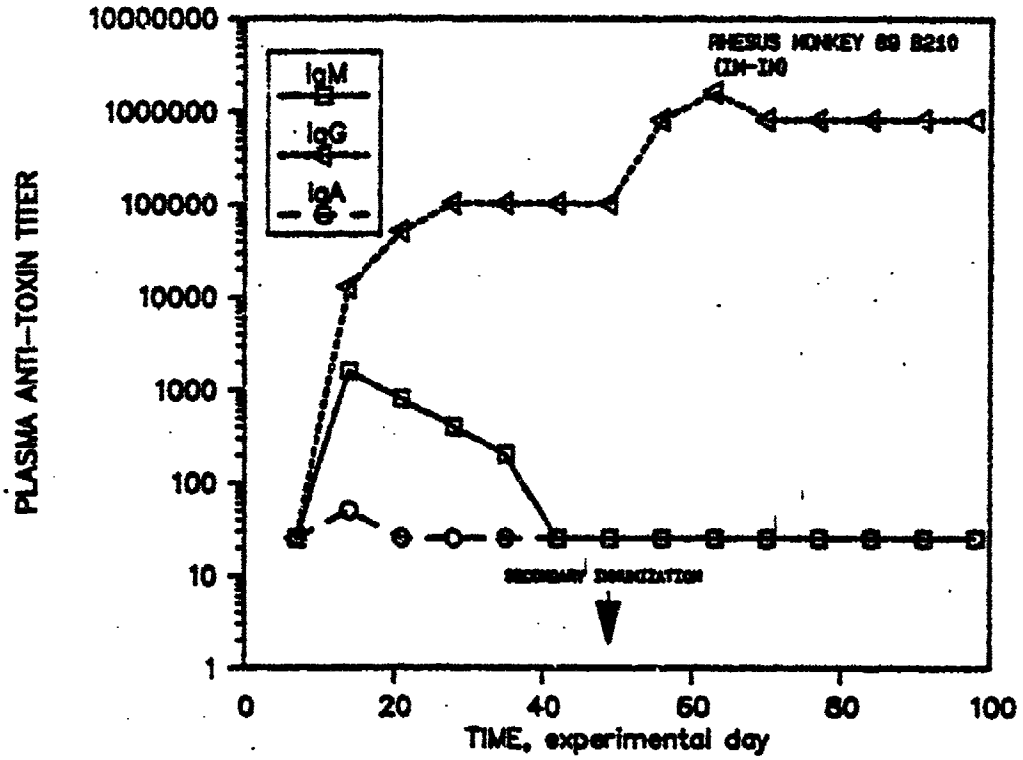


Figure 13. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 89 B210: Primary immunization (Day 0)—100 micrograms microencapsulated SEB toxoid by IM injection. Secondary immunization (Day 49)—100 micrograms of microencapsulated SEB toxoid by IM injection.

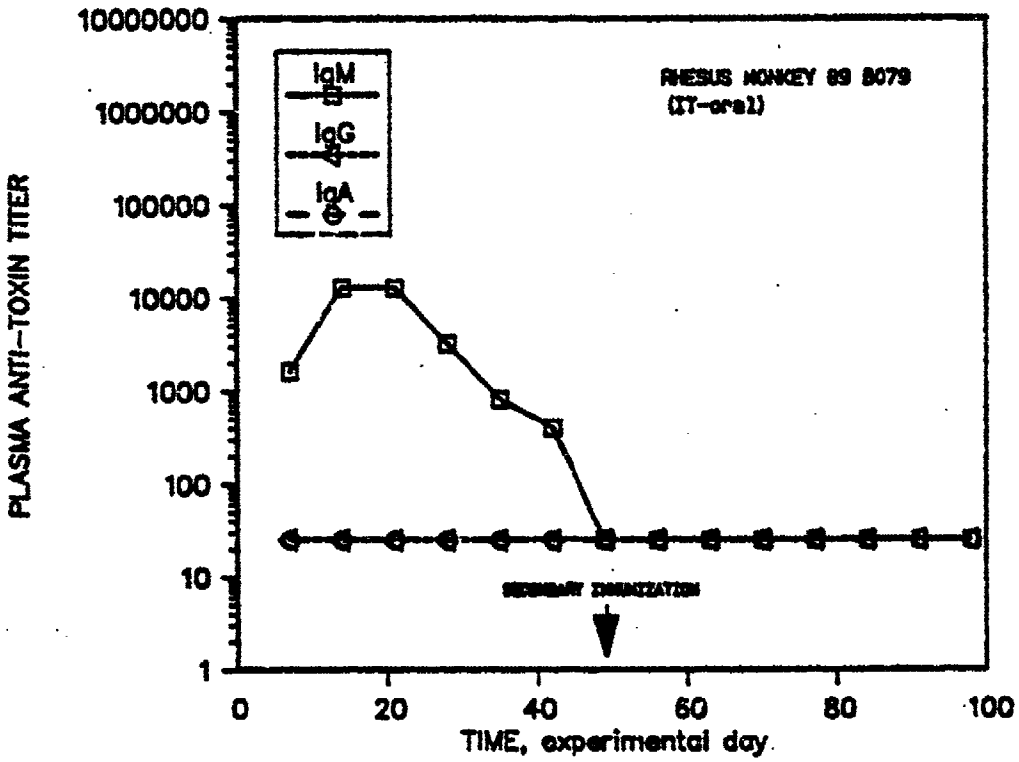


Figure 14. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 89 B079: Primary immunization (Day 0)—100 micrograms microencapsulated SEB toxoid by IT instillation. Secondary immunization (Day 49)—1.0 mg of microencapsulated SEB toxoid by oral gavage.

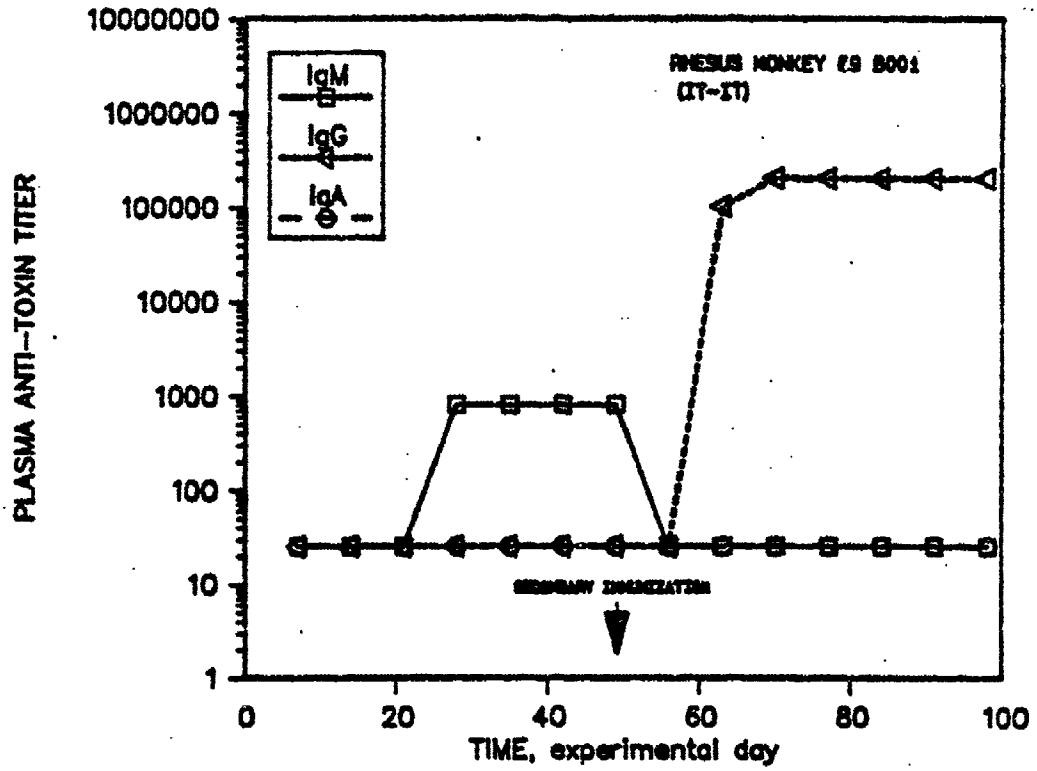


Figure 15. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 89 B001: Primary immunization (Day 0)—100 micrograms microencapsulated SEB toxoid by IM injection. Secondary immunization (Day 49)—100 micrograms of microencapsulated SEB toxoid by IT instillation.

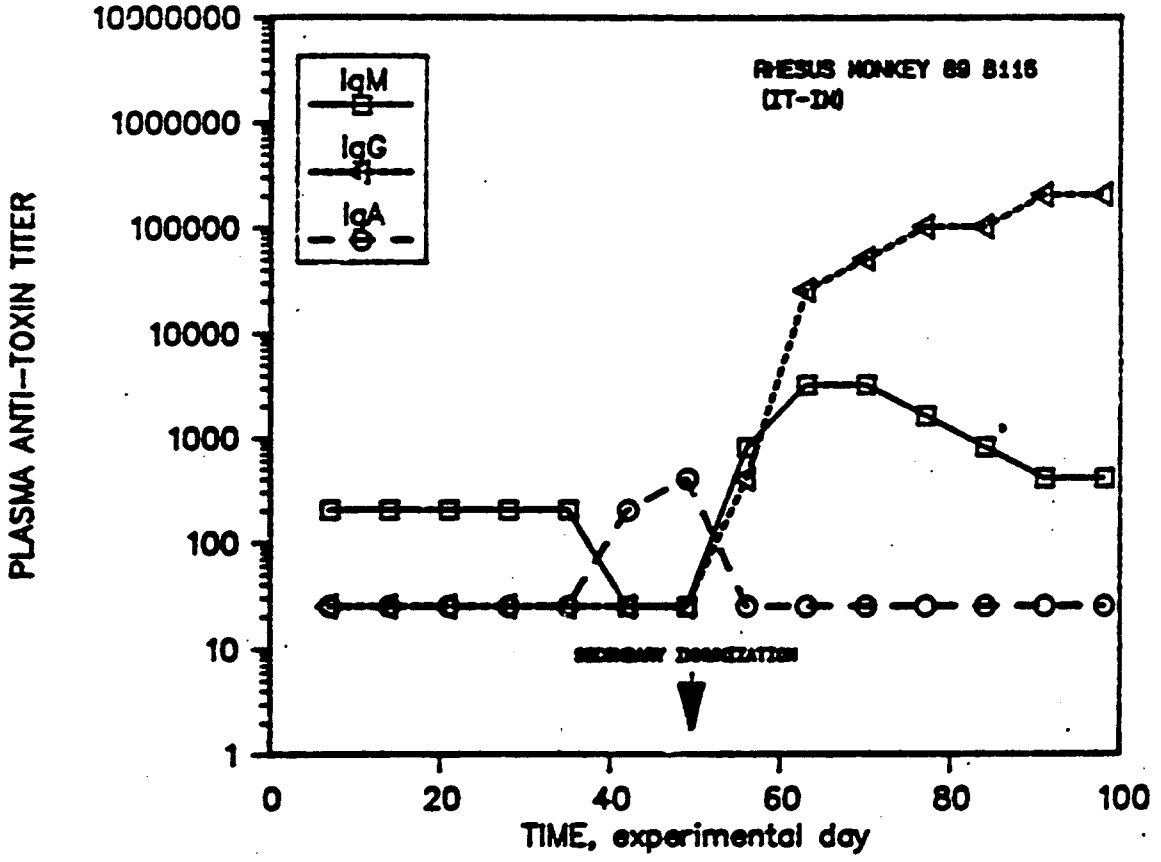


Figure 16. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 89 B116: Primary immunization (Day 0)—100 micrograms microencapsulated SEB toxoid by IT instillation. Secondary immunization (Day 49)—100 micrograms of microencapsulated SEB toxoid by IM injection.

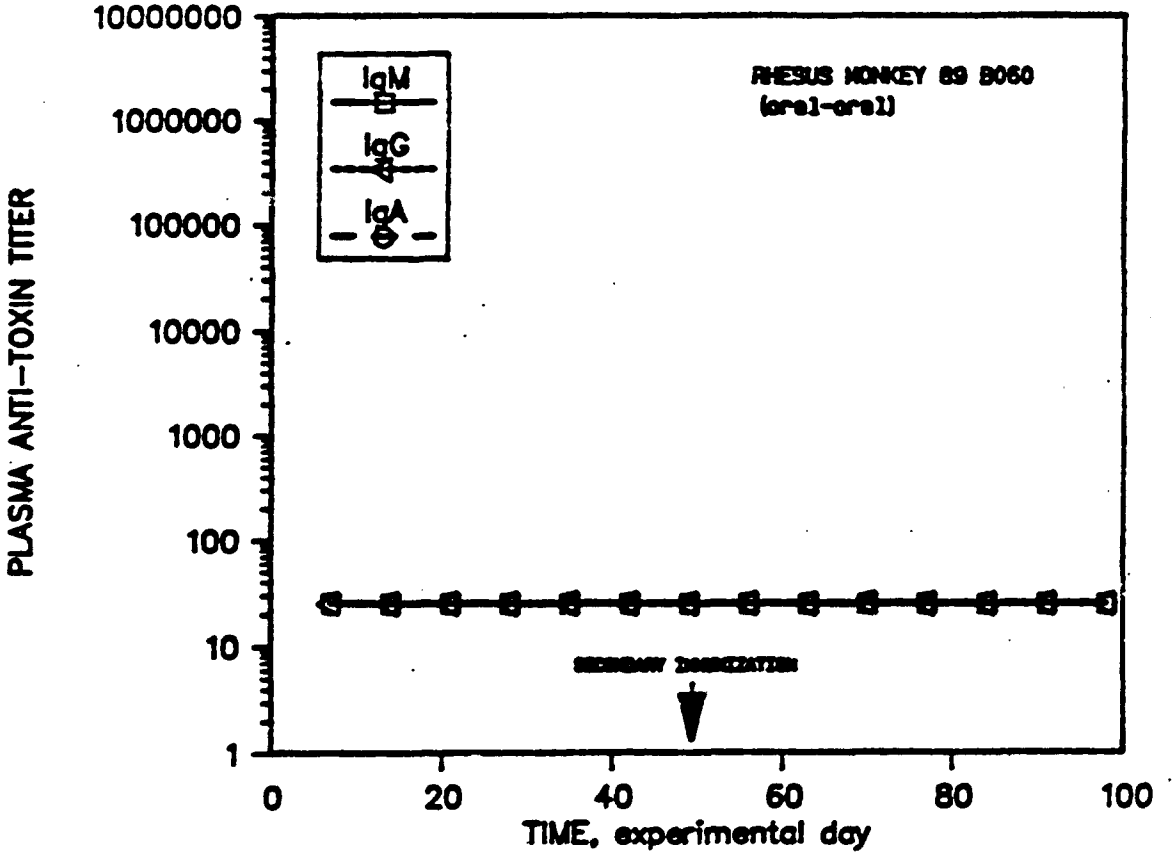


Figure 17. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 89 B060: Primary immunization (Day 0)—1.0 mg microencapsulated SEB toxoid by oral gavage. Secondary immunization (Day 49)—1.0 mg of microencapsulated SEB toxoid by oral gavage.

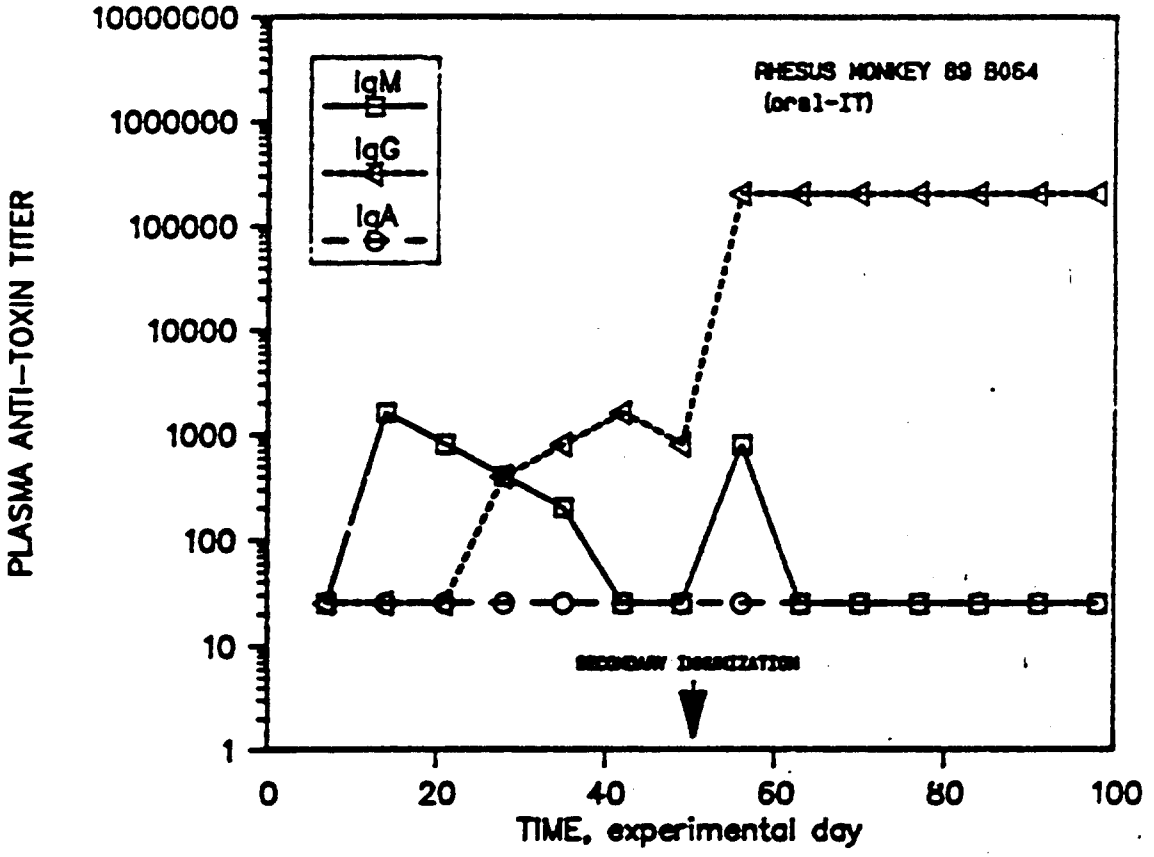


Figure 18. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 89 B064: Primary immunization (Day 0)—1.0 mg microencapsulated SEB toxoid by oral gavage. Secondary immunization (Day 49)—100 micrograms of microencapsulated SEB toxoid by IT instillation.

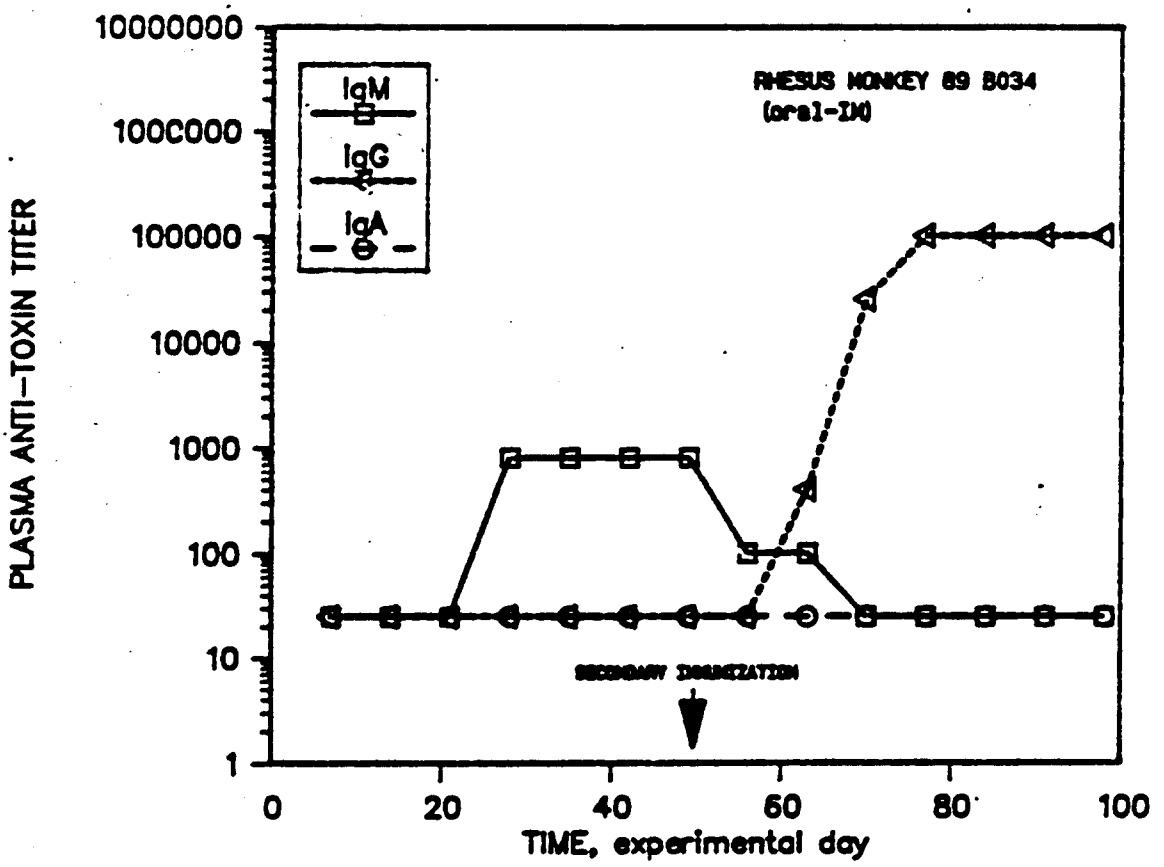


Figure 19. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 89 B034: .Primary immunization (Day 0)—1.0 mg microencapsulated SEB toxoid by oral gavage. Secondary immunization (Day 49)—100 micrograms of microencapsulated SEB toxoid by IM injection.

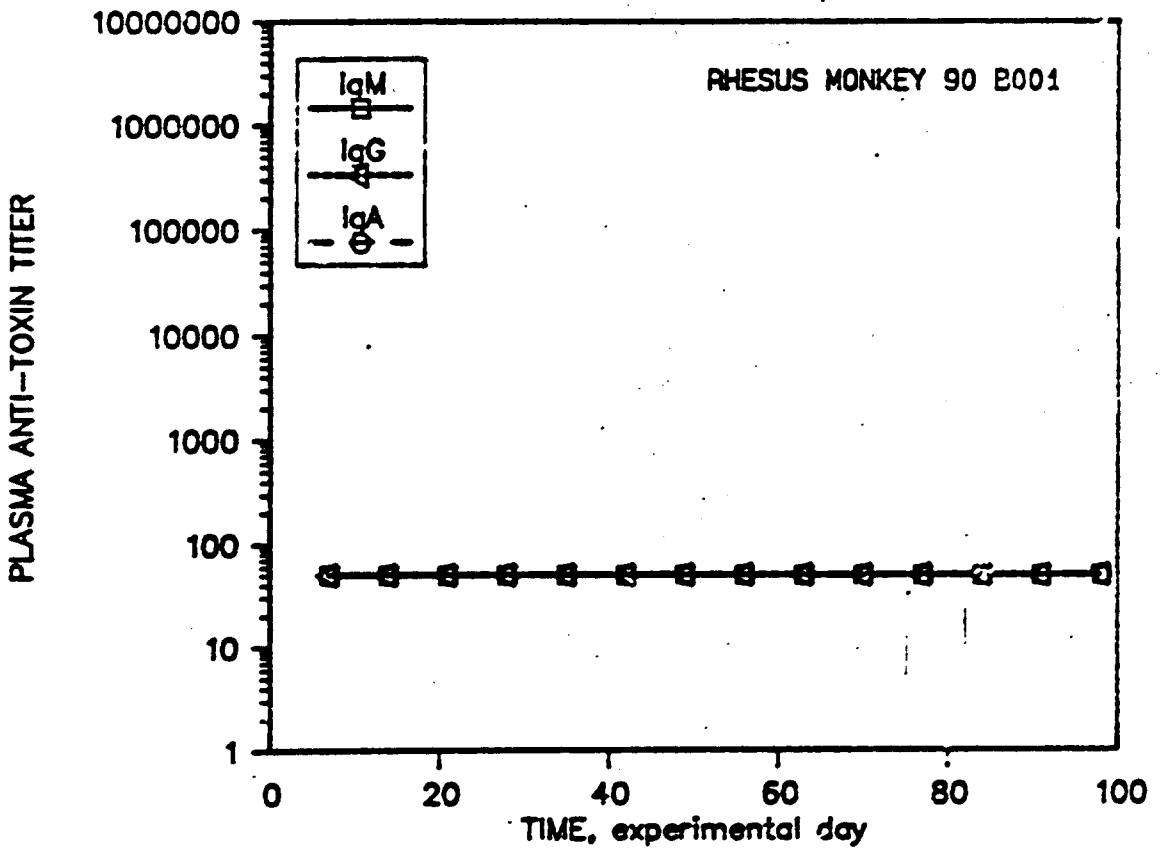


Figure 20. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 90 B001: Non-immunized control.

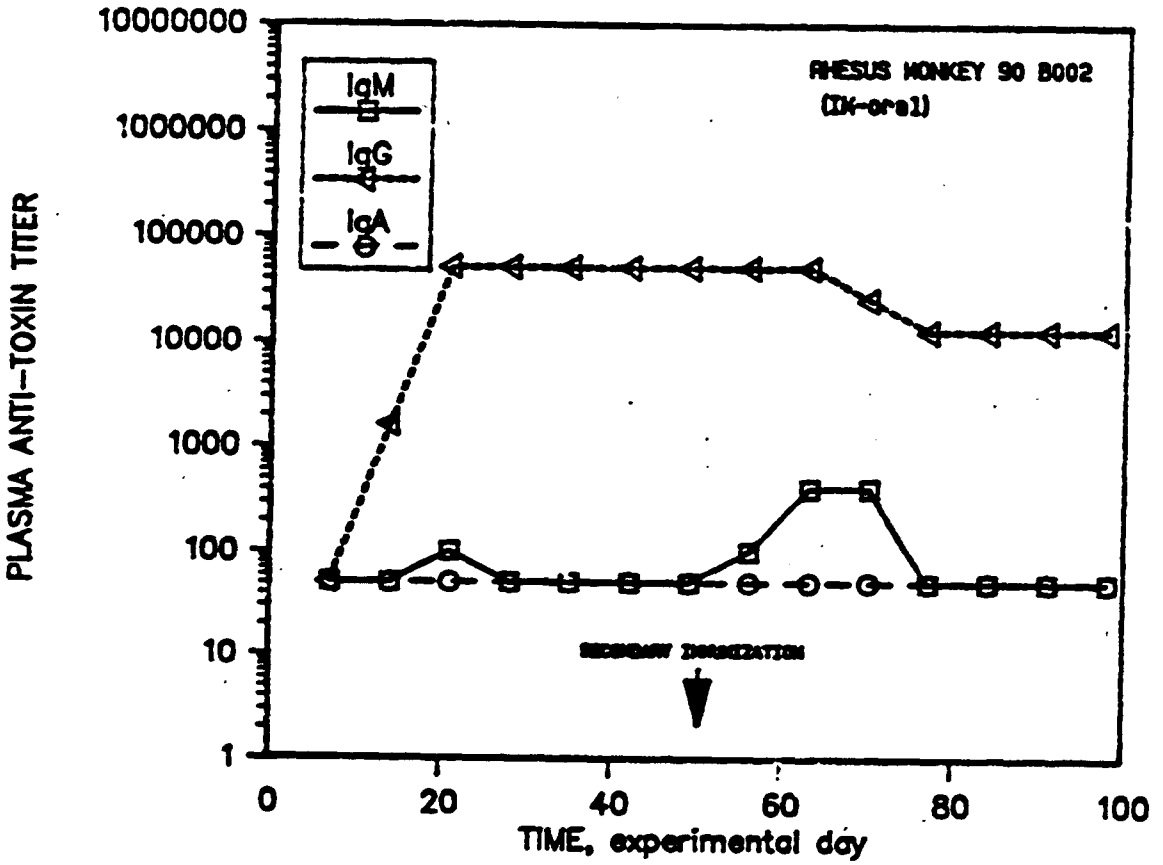


Figure 21. Plasma IgM, Ig6, and IgA anti-SEB toxin titers obtained from rhesus Monkey 90 B002: Primary immunization (Day 0)—100 micrograms micro··ncapsulated SEB toxoid by IM injection. Secondary immunization (Dav 49)—1.0 rng of microencapsulated SEB toxoid by oral gavage.

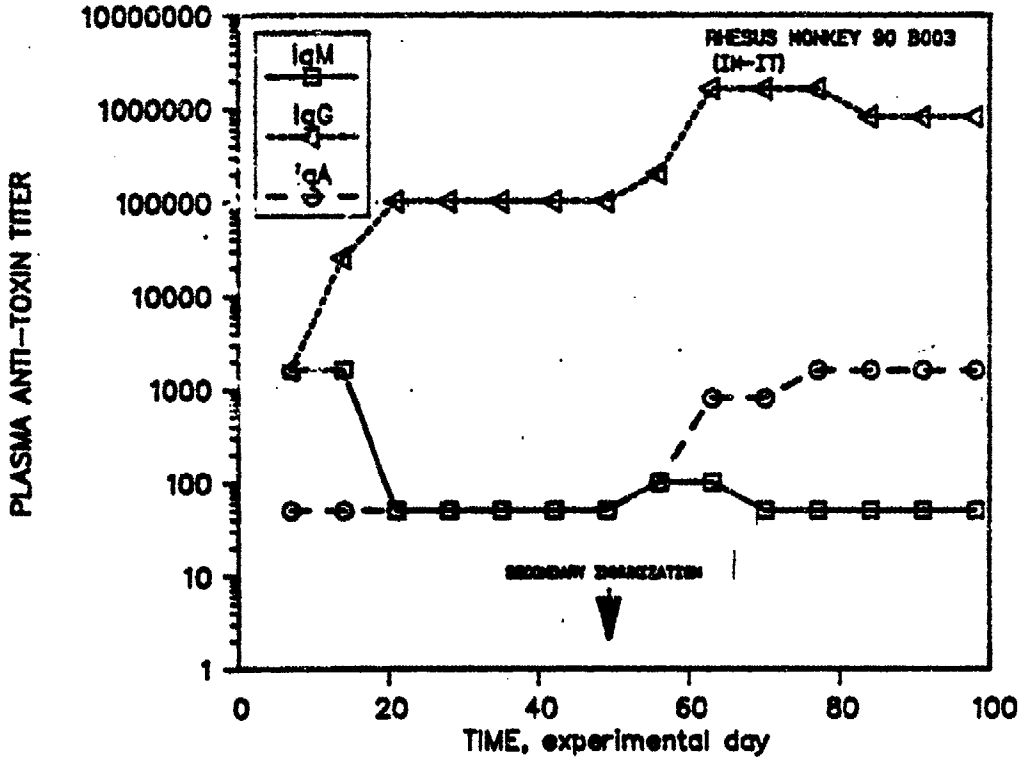


Figure 22. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 90 B003: Primary immunization (Day 0)—100 micrograms microencapsulated SEB toxoid by IM injection. Secondary immunization (Day 49)—100 micrograms of microencapsulated SEB toxoid by IT instillation

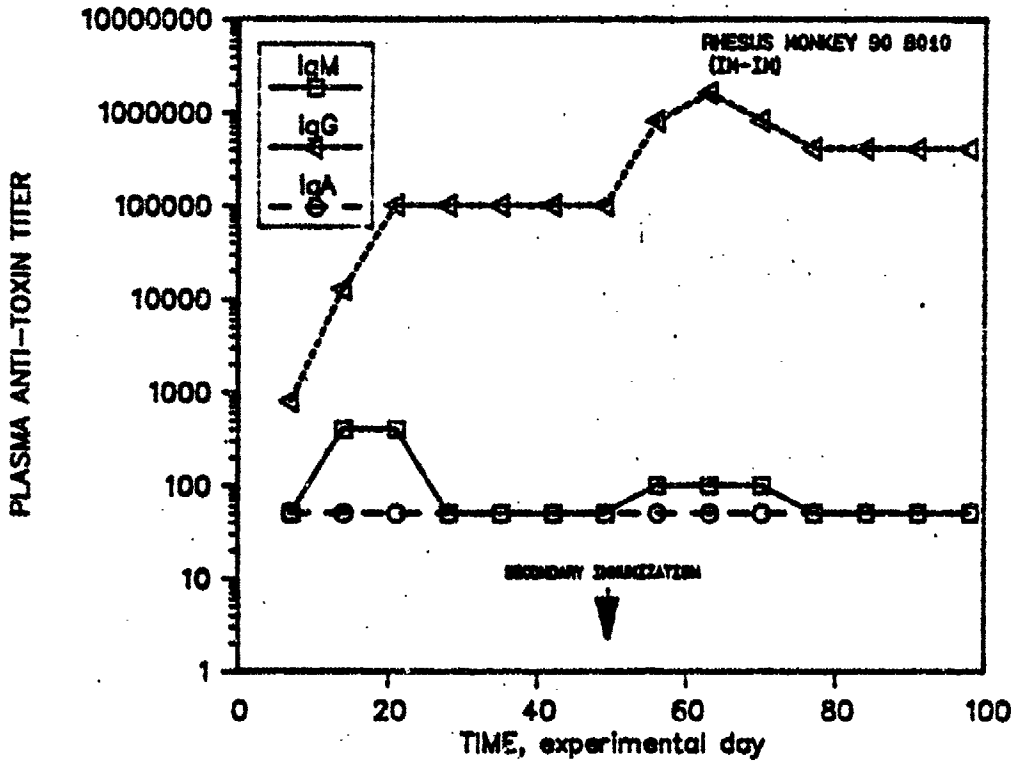


Figure 23. Plasma IgM, IgG, and IgA anti-SEC toxin titers obtained from rhesus Monkey 90 B010: Primary immunization (Day 0)—100 micrograms microencapsulated SEB toxoid by IM injection. Secondary immunization (Day 49)—100 micrograms of microencapsulated SEB toxoid by IM injection.

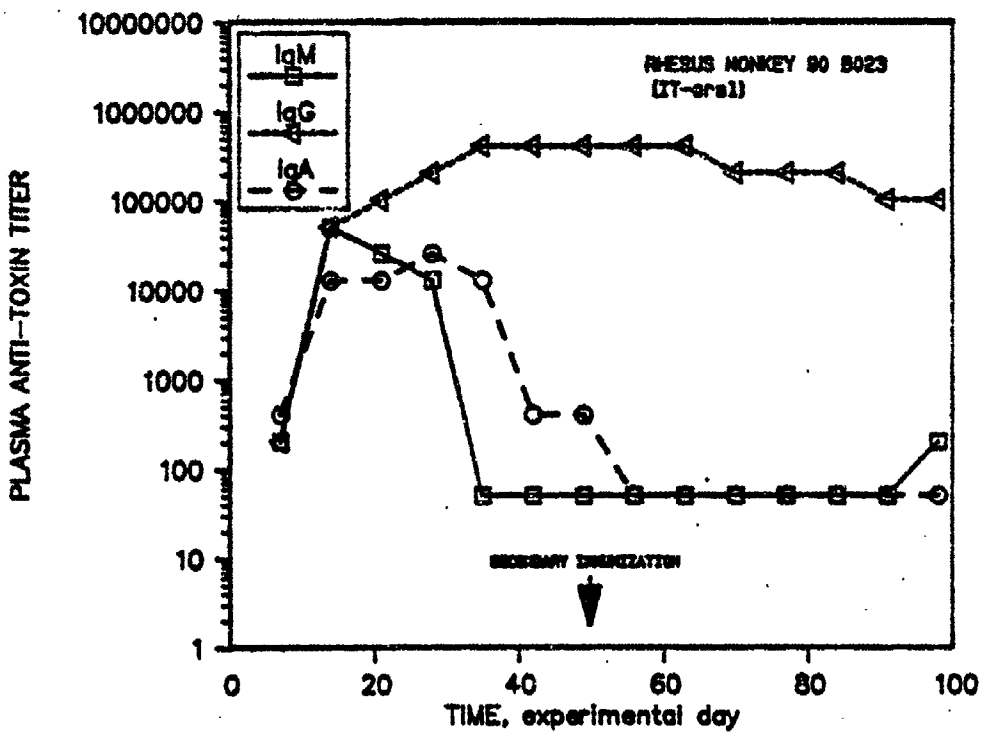


Figure 24. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 90 B023: Primary immunization (Day 0)—100 micrograms microencapsulated SEB toxoid by IT instillation. Secondary immunization (Day 49)—1.0 mg of microencapsulated SEB toxoid by oral gavage.

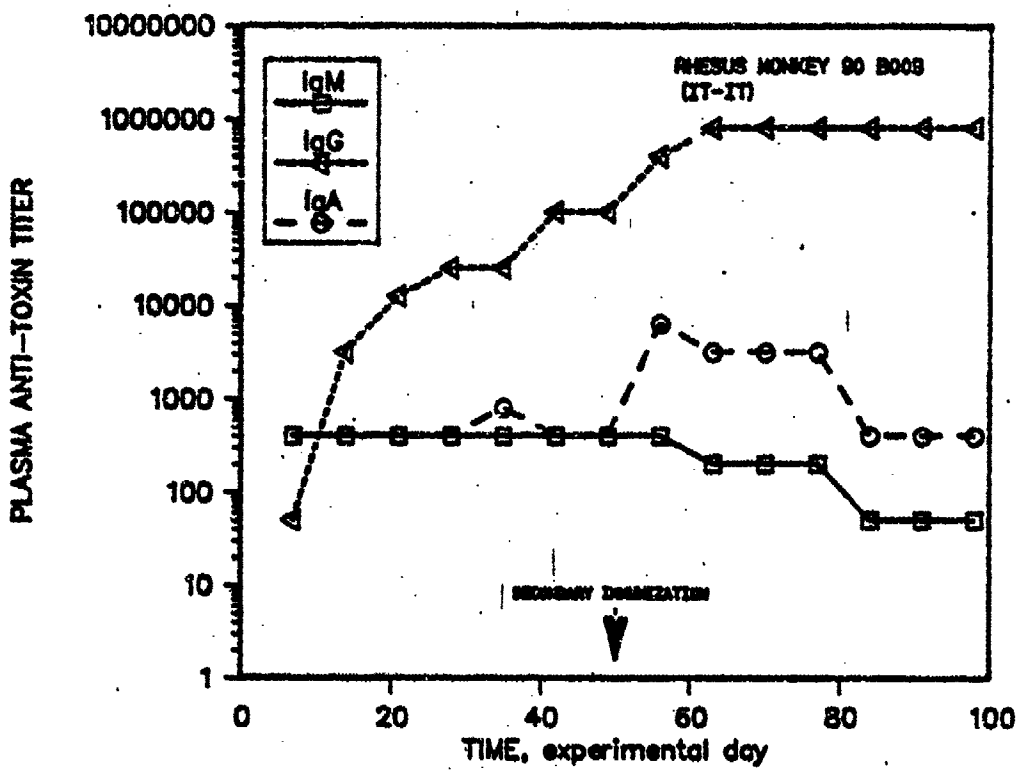


Figure 25. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 90 B009: Primary immunization (Day 0)—100 micrograms microencapsulated SEB toxoid by IT instillation. Secondary immunization (Day 49)—100 micrograms of microencapsulated SEB toxoid by IT instillation.

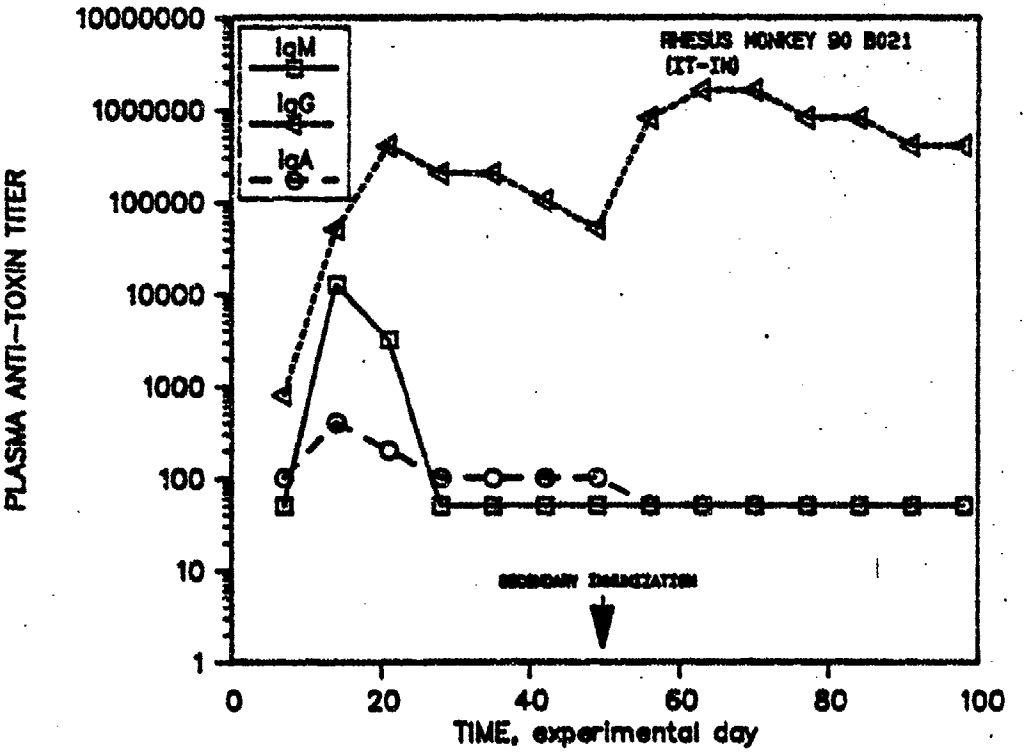


Figure 26. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 90 B021: Primary immunization (Day 0)—100 micrograms microencapsulated SEB toxoid by IT instillation. Secondary immunization (Day 49)—100 micrograms of microencapsulated SEB toxoid by IM injection.

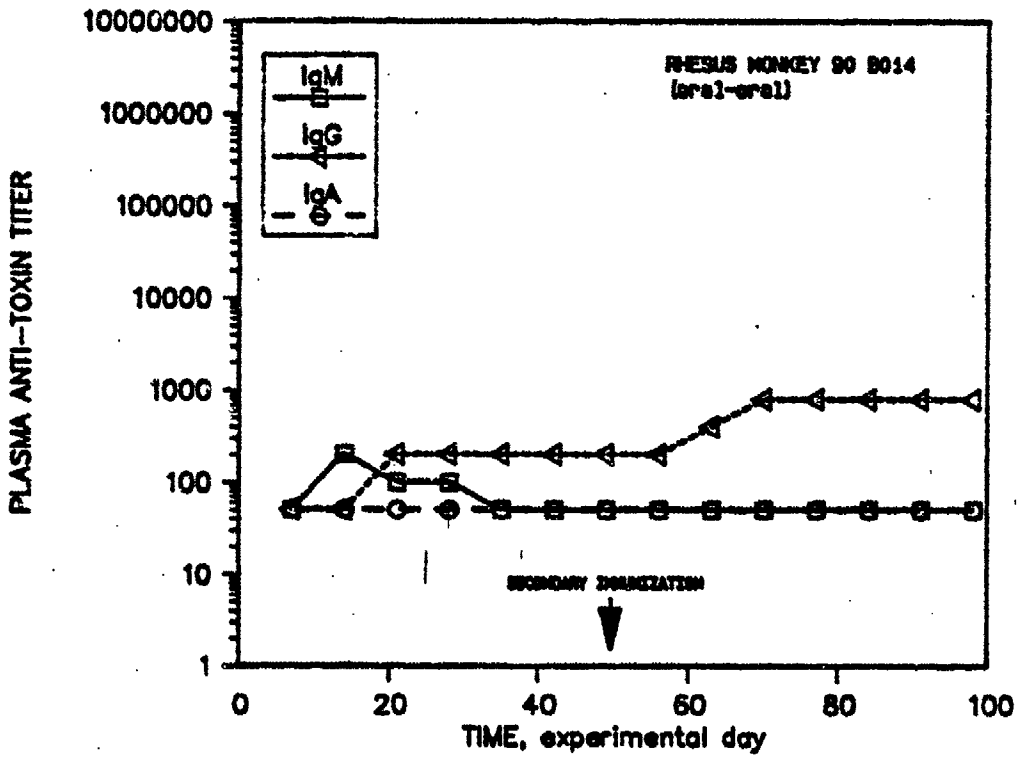


Figure 27. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 90 B014: Primary immunization (Day 0)—1.0 mg microencapsulated SEB toxoid by oral gavage. Secondary immunization (Day 49)—1.0 mg of microencapsulated SEB toxoid by oral gavage.

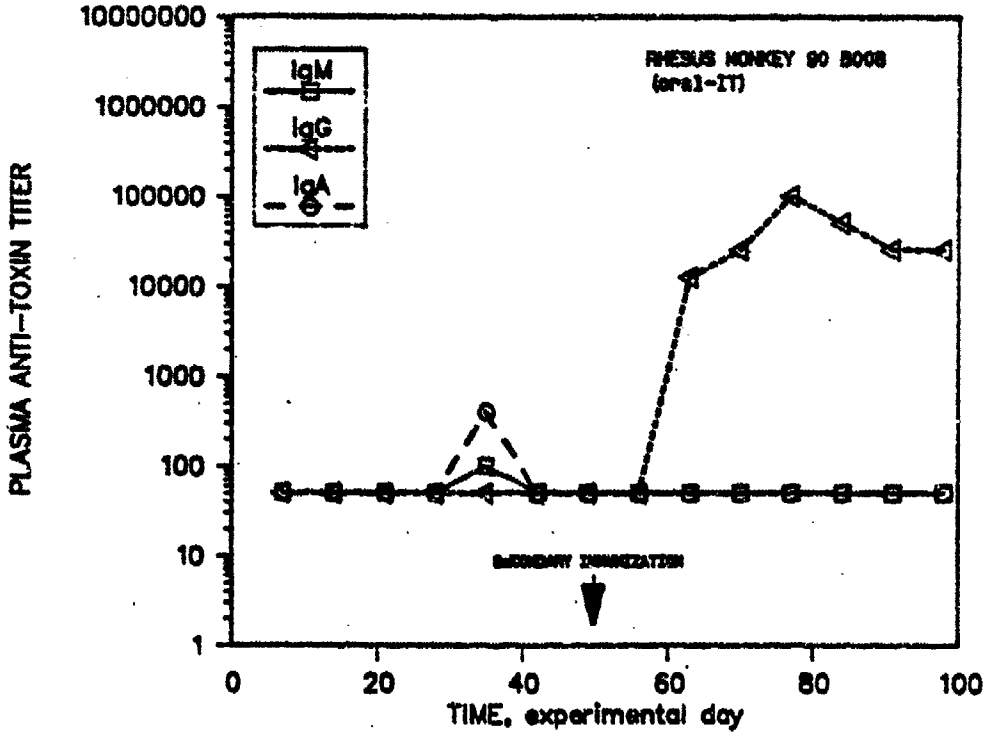


Figure 28. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 90 B008: Primary immunization (Day 0)—1.0 mg microencapsulated SEB toxoid by oral gavage. Secondary immunization (Day 49)—100 micrograms of microencapsulated SEB toxoid by IT instillation.

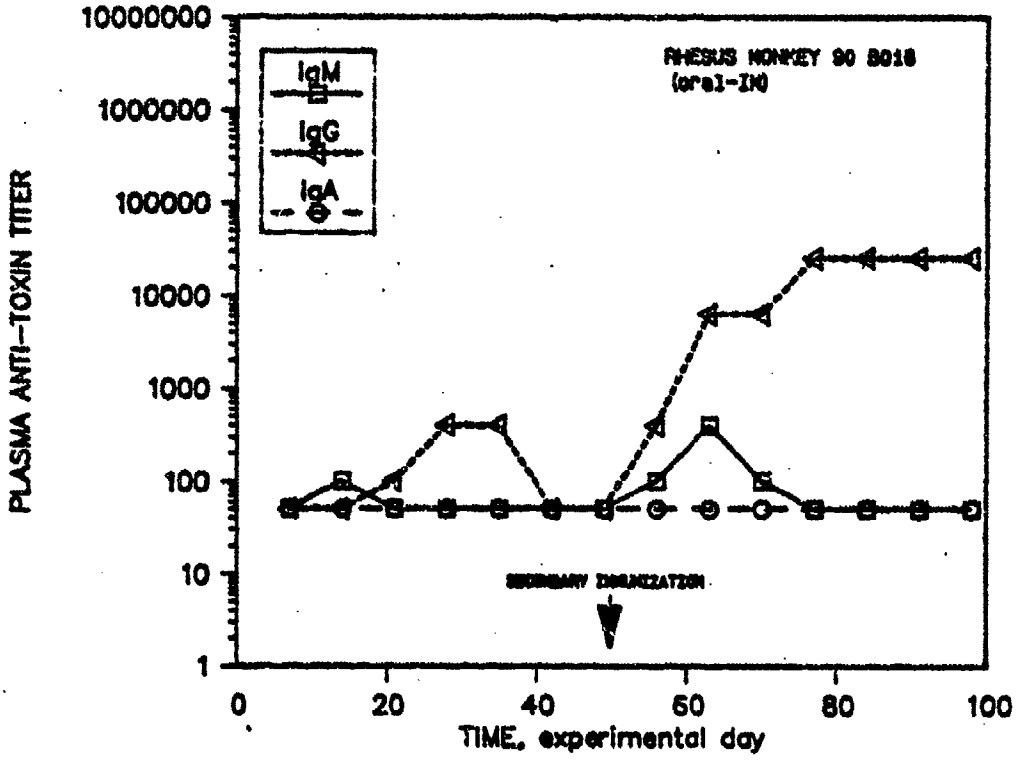


Figure 29. Plasma IgM, IgG, and IgA anti-SEB toxin titers obtained from rhesus Monkey 90 8018: Primary immunization (Day 0)—1.0 mg microencapsulated SEB toxoid by oral gavage. Secondary immunization (Day 49)—100 micrograms of microencapsulated SEB toxoid by IM injection.

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