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Efficacy and Mode of Action of Immune Response Modifying Compounds Against Alphaviruses and Flaviviruses

Annual Report

Page S. Morahan, Ph.D.
Margo Brinton, Ph.D.
Angelo J. Pinto, Ph.D.

December 1987

Support by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-86-C-6117

The Medical College of Pennsylvania
Department of Microbiology and Immunology
3300 Henry Avenue
Philadelphia, PA 19129

SECURITY CLASSIFICATION OF THIS PAGE

AD-A205252

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: The Medical College of Pennsylvania; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): Department of Microbiology and Immunology, 3300 Henry Avenue, Philadelphia, PA 19129	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-86-C-6117
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO. 3M1-	TASK NO.	WORK UNIT ACCESSION NO.
62770A	62770A871	AG	370

11. TITLE (Include Security Classification): (U) Efficacy and Mode of Action of Immune Response Modifying Compounds Against Alphaviruses and Flaviviruses

12. PERSONAL AUTHOR(S): Page S. Morahan, Margo Brinton, Angelo J. Pinto

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 1/1/87 TO 12/31/87	1987 December	67

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
06	15		Keywords: Immunomodulators, herpes simplex virus, Banzi virus, Venuezualean encephalitits, Caraparu virus, interferon, C. parvum, CL246,738, ampligen, MVE-2, (see back)
06	13		

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

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(Continued on other side)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mary Frances Bostian	301/663-7325	SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

18. Keywords (continued)

Macrophage activation, Natural Killer cells, Antiviral therapy

19. Abstract (continued)

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SUMMARY

This current study demonstrates that several immunomodulators protect mice against challenge with Semliki forest alphatogarvirus (SFV), Banzi flavivirus (BV), Caraparu bunyavirus (CV), and herpes simplex virus (HSV-2). Prophylactic treatment with maleic anhydride divinyl ether copolymer, C. parvum, CL246,738, Ampligen, recombinant murine gamma interferon (rMuIFN-G) and recombinant human alpha A/D interferon (rHuIFN-A A/D) reduced mortality and increased survival time significantly. Early therapeutic treatment with some agents (CL246,738, rMuIFN-G, rHuIFN-A A/D, and Ampligen) was also effective. Three pyrimidinones and various microbially derived compounds were also found to have antiviral activity. In contrast, human recombinant alpha tumor necrosis factor (rHuTNF-A) and human macropahge colony stimulating factor (rHuCSF-M) were ineffective against the viruses tested. Among the active immunomodulators, broad spectrum antiviral activity was observed; however, there were differences in virus sensitivity with SFV and BV being the most sensitive and CV being the least sensitive to immunomodulator treatment. In contrast, CV was most sensitive to treatment with the synthetic nucleoside analogue ribavirin. Many of the compounds activated macrophages and natural killer (NK) cell functions however, no unified immunomodulatory antiviral mechanism was identified. Selective depletion methods for MØ and NK cells were evaluated for potential use in assessing whether the antiviral activity of immunomodulators requires the presence of MØ or NK cell antiviral activity. (RW)

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

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

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INTRODUCTION

This research is directed at two of the areas of interest for developmental research on immune modifying compounds against viruses of military importance.

1. Evaluate compounds for modulation of specific and nonspecific immune responses in normal B6C3F1 female mice. We are using drug treatment regimens that have been shown to be effective in the in vivo antiviral tests by us and others. We have focused on in vitro/ex vivo tests for intrinsic and extrinsic macrophage antiviral activity, macrophage activation, and natural killer cell activity.

2. Establish the efficacy of prophylactic and/or therapeutic treatment with immunomodulators alone and in combination with antiviral drugs against alphavirus, flavivirus, bunyavirus and herpesvirus infections in vivo. We are evaluating and characterizing the antiviral efficacy in normal mice, and we are developing systems to evaluate a few regimens of particular interest in mice that have been selectively depleted of various nonspecific effector cell populations.

BACKGROUND

The need for better control of the "exotic" RNA viruses which are members of the Alphatogaviridae, Flaviviridae, Bunyaviridae, and Arenaviridae is well documented. Complete control will undoubtedly require a multifaceted approach, including better insect and rodent vector control, effective vaccines, and development of safe and effective prophylactic/therapeutic treatment. Development of antiviral treatment of RNA viruses has lagged behind drug development for DNA herpesviruses, where we now have the second generation of active nucleoside analogues (7,21). Ribavirin has been shown to be effective for a few RNA viruses in both animal models and clinical settings (22,23). Considerable research in recent years has also documented that many RNA viruses are inhibited by a variety of immunomodulators, including interferon (7,13,14,29,31,52,53,56,70). Importantly, combinations of two different nucleosides or immunomodulators have clearly been shown to produce synergistic effects (18,19). Most of these studies have been performed only in vitro (21,36). There is evidence, however, of synergy in vivo for ribavirin combined with the interferon inducer poly IC-LC against Rift Valley fever virus (RVF) infection in mice (25,26,28), and for ribavirin and specific antibody against Lassa fever virus in monkeys (24). Antiviral activity against RVF in mice can also be enhanced if nucleosides such as ribavirin or immunomodulators such as muramyl dipeptide (MDP) are delivered in liposomes (7,16,25,27,30).

Neither the predominant mechanisms of action of these various drug combinations, nor the optimum treatment regimens (dose, schedule, route) have yet been established. It has been well documented that immunomodulators can exert "yin-yang" effects on host resistance varying with the compound injection dose, route and schedule in relation to virus or tumor challenge (20,49,54,60). Nucleosides have toxic properties (8,9) and can also alter the lymphoreticular/hematopoietic system and thus alter the toxicity or the

protective action of immunomodulators (14). Thus, systematic evaluation of antiviral efficacy and immunomodulatory activity of promising compounds and compound combinations is necessary before the best clinical treatment can be developed.

For these reasons, the current study has evaluated different regimens of immunomodulator treatment of antiviral efficacy against diverse RNA viruses and has attempted to correlate antiviral protection with antiviral effector mechanisms. Specifically, we have focused on drug-induced changes in macrophage (MØ) activation, as assessed by antitumor activity and ectoenzyme phenotype, and natural killer cell (NK) activity, mechanisms which have been well documented to be important in resistance to virus infections (2,3,43,44,63,71) Our results demonstrate that several immunomodulators are effective antiviral agents in vivo. Thus, these drugs may be useful in prophylactic or early therapeutic treatment of several severe viral infections. Whether the antiviral mechanism(s) of action is related to the effects on the natural immune system will be a major thrust of our future studies.

RATIONALE

A variety of potentially useful immunomodulators are being characterized for immunomodulatory activity in the B6C3F1 female mouse animal model that is widely accepted for immunotoxicological and immunopharmacological preclinical evaluations. A battery of standardized nonspecific immune function assays is being performed. We are focusing especially on MØ and NK cell activation (47). The antiviral activity of MØ against four classes of RNA viruses (alpha-, flavi-, bunya- and arena-viruses) is being studied to establish if MØ exert broad spectrum antiviral activity through common antiviral mechanisms. The study of novel immunomodulators in standardized assays, in comparison with "classic" immunomodulators, allows assessment of the most probable effective immunomodulatory mechanism of the new compounds.

The second focus of the present research is the establishment of the antiviral efficacy of immunomodulators against a variety of exotic RNA virus infections. For comparison, antiviral efficacy against HSV-2 infection is used as a "gold standard", because the mechanisms of natural host resistance and protective immunomodulatory treatment against HSV have been extensively characterized. A spectrum of RNA virus infections has been selected for study, in order to establish if broad spectrum protection can be produced against viruses that possess quite different replication and viral pathogenesis patterns. The models selected include intraperitoneal infections with Semliki forest strain L10 alphavirus, Banzi flavivirus, and Oriboca or Caraparu bunyaviruses. These viruses produce significant mortality in adult mice after i.p. infection, but can be worked with under Class II biohazard conditions.

In summary, the rationale for this research is that: (i) better methods are needed for treatment of RNA virus infections; (ii) immunomodulation provides a potent new modality of antiviral prophylaxis and therapy; (iii) but, immunomodulation can also produce adverse effects. Thus, there is a need for systematic evaluation of immunomodulators against diverse viral infections, together with more precise delineation of their mechanisms of action on

nonspecific immune effector cells and immunoregulatory networks under carefully controlled standard systems.

EXPERIMENTAL METHODS

Mice. Virus free, barrier raised, 6 week old female B6C3F1 mice were purchased from Ace Animals Inc. or Taconic Farms, shipped in filter crates and housed in autoclaved micro-isolator cages (MCP) or PLAS - LAB isolator chambers (Wistar). Usually, two mice from each shipment were bled on arrival, two more at one week, and periodically thereafter for testing to ensure no inapparent viral infections had occurred. Mouse sera were tested for sero-conversion to MHV and Sendai viruses by the ELISA test (Biocon Labs, Rockville, MD). No sero-conversion for these viruses has been observed in the last 24 months.

Immunomodulators: Table 1 lists the immunomodulators used in this study. C. parvum (Burroughs Wellcome Co., Research Triangle Park, NC) was injected into mice i.p. at 35 mg/kg 7 days prior to cell harvest or infection of mice. MVE-2 (Hercules, Inc., Wilmington, DE) was dissolved in phosphate-buffered saline to a final inoculation concentration of 50 mg/kg and was administered i.p. to mice 1 day prior to virus infection. TDM and MPL (Ribi Immunochem Research Inc., Hamilton, MT) were solubilized in 2% squalene at 56°C and injected i.p. rMuIFN-G (courtesy of Genentech, South San Francisco, CA), rHuIFN-A A/D (courtesy of Hoffman La Roche, Nutley, NJ), rHuTNF-A (courtesy of Genentech) rHuIL-1 B (courtesy of Dr Phil Simon, Smith Kline French Research Laboratories), and rHuCSF-M (courtesy of Dr. Peter Ralph, Cetus Corporation, CA) were all injected i.p. in a vehicle consisting of phosphate-buffered saline containing 0.2% bovine serum albumin (BSA). Each time an IFN was prepared for injection, it was titered simultaneously for antiviral activity. Ampligen (provided to USAMRIID by Dr. Paul Tso, Johns Hopkins, Baltimore, MD) was dissolved in physiological saline, heated at 67°C for 16 hours then at 37°C for 1 hour before being injected i.p. CL246,738 (courtesy of Lederle, Pearl River, NY) was prepared in distilled water and administered per os. The three pyrimidinones, ABPP, AIPP and ABMP were received courtesy of Dr. Harold Renis, Upjohn Co., Kalamazoo, MI. All were suspended in 1% carboxy-methylcellulose (CMC). To ensure a uniform suspension, pyrimidinone preparations were vortexed vigrously just prior to i.p. inoculation. GE-132 was ground and suspended in 1% CMC (for per os. administration) or Tween 80 (for i.p. administration).

Viruses. Each virus pool was prepared somewhat differently, in order to maximize the titer of virus produced. The in vitro and in vivo titers of the various viruses are presented in Table 2. Herpes simplex virus type 1 (HSV-1 Kos) and type 2 (HSV-2 MS) were prepared in either secondary rabbit kidney fibroblasts or Vero cells, by infecting cells with a low multiplicity of infection (m.o.i.) , and harvesting the cultures when more than 75% of the cells showed cytopathic effect (CPE) (45). The pools consisted of cell-associated virus that was clarified of cellular debris by low speed centrifugation. Both HSV-1 and HSV-2 were titered on Vero cells with 2% methylcellulose overlay, and the titers were about 2×10^8 plaque forming units (PFU/ml) for HSV-1 and 8×10^6 PFU/ml for HSV-2.

Pools of alphaviruses, flaviviruses, and bunyaviruses were made in newborn CD-1 mice. In all cases two to four day old mice were inoculated with 0.02 ml of virus by the intracerebral route. When moribund, the mice were sacrificed by decapitation and their brains or livers removed and frozen on dry ice. Subsequently, clarified 10% (wt/vol) tissue homogenates were made, aliquoted, and stored frozen at - 70°C.

A stock of Pichinde virus (strain Co An 3739) propagated in Vero cells was obtained from Dr. Pat Repik. A pool was prepared by infecting Vero cells with a m.o.i. of 0.0001, harvesting cell associated virus after three days of incubation by distilled water lysis and one freeze thawing to disrupt cells, followed by clarification of the fluid by low speed centrifugation and storage at -70°C. A suckling mouse brain pool (SMB pool 1, October 1977) of Semliki forest virus (SFV) L10 strain was received from USMRIID, diluted 10-fold and frozen at - 70°C. It has a titer of 6.8×10^7 PFU/ml on BHK21 cells, and an i.p. LD_{50} of $10^{7.2}$, or about 1 PFU/LD50 in adult B6C3F1 mice.

The flavivirus, Yellow Fever virus, 17D vaccine strain, was obtained from Connaught Laboratories. Mice were inoculated with 2×10^2 PFU/mouse and were moribund by 7 days after infection. A brain pool was prepared and contained a titer of 1.6×10^7 PFU/ml as assayed on BHK21 cells. A seed stock of the flavivirus, Banzi virus, strain SA M336, mouse passage 9, was obtained from Dr. Shope. Mice were inoculated with a 1:2 dilution of this stock and were sacrificed 3 days after infection. The brain pool prepared contained an infectivity titer of 2.0×10^8 PFU/ml as assayed by plaque titration on BHK 15 cells.

A seed stock of a second mouse passage of the bunyavirus, Caraparu, strain Be AN 3999, was obtained from Dr. Shope, Mice were inoculated intracerebrally with a 1:100 dilution of this stock and sacrificed 48 hr after infection. The livers were removed and used to prepare a clarified 10% (wt/vol) liver homogenate. No plaques were obtained with this virus on BHK-21, BHK-15 or MK2 cell monolayers. The pool had an LD_{50} of $10^{2.33}$ in adult CD-1 mice inoculated by the i.p. route. A seed stock of the bunyavirus, Oriboca, strain BeAn 17, mouse passage 12, was obtained from Dr. Shope. Mice were inoculated with a 1:100 dilution of this stock and sacrificed 48 hr after infection. A 10% liver virus pool was prepared and had an infectivity titer of 4×10^6 PFU/ml as assayed on BHK-21 cells.

Target Cell Lines. Two different cell lines were used in this study. These were: Lewis lung (LL), a carcinoma derived from spontaneous tumors in C57BL/6 mice (65); and YAC-1, a T cell lymphoma derived from the A/SN mouse (17). LL was maintained as monolayers in 25cm^2 plastic flasks, with Eagle's minimum essential medium (EMEM) (Sigma, St. Louis, MO) supplemented with 10% FBCS (Flow Laboratories, McLean, VA), 2% MEM vitamins (Flow), 1 mM sodium pyruvate, 1% essential amino acids (Flow), 1% non-essental amino acids (Flow) and 25 mM HEPES buffer (Sigma). For convenience, this culture medium hereafter will be called complete EMEM. YAC-1 was maintained in stationary suspension culture in 25cm^2 plastic flasks with RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% FBCS (Flow), and 25 mM HEPES buffer (Sigma). For convenience, this culture medium hereafter will be called complete RPMI.

Peritoneal MØ Preparation. MØ were harvested by flushing the peritoneal cavity

with 5ml of cold Dulbecco's phosphate-buffered saline (DPBS) (Gibco) containing 2 units/ml preservative-free heparin (Invenex Laboratories, Melrose Park,IL), and 50ug/ml gentamicin (Gibco). The peritoneal cells were counted in a Model ZM Coulter Counter (Hialeah, FL), centrifuged at 250 x g for 10 minutes at 5°C and resuspended to a concentration of 2.5 x 10^6 cells/ml in complete EMEM containing 50ug/ml gentamicin. Slides for cell differentials were prepared in a Cytospin (Shandon-Southern, Sewickley, PA) and were stained with a modified Wright's stain (Geometric Data, Wayne, PA).

Preparation of Spleen Cells. Spleens were removed, trimmed of fat and placed in 10ml manual glass tissue grinders containing 5ml cold RPMI 1640. Spleen cells were dispersed into the RPMI 1640 by gentle grinding, and the resulting suspension was transferred to a 17 x 100 mm round bottom plastic centrifuge tube. After an initial wash, contaminating red blood cells were removed by resuspending the cell pellet in distilled water for 20 seconds followed by an equal volume of 2 x DPBS. Cells were then washed again at 250 x g for 10 minutes before being resuspended to 2 x 10^7 cells/ml in complete RPMI.

Preparation of Kupffer cells (KC). Mice were sacrified by cervical dislocation, their abdominal skin retracted and their thoracic and peritoneal cavities exposed. A sterile 25-gauge 1 inch needle butterfly infusion set was inserted into the inferior vena cava to the level of the renal artery. To wash the liver free of blood the liver was perfused with 10 ml Hanks' balanced salt solution (HBSS), Ca^{++}, Mg^{++} free, containing 25 mM HEPES buffer and 1 mM EDTA. To allow for hepatic efflux, the portal vein was cut just prior to perfusion. When this perfusion was properly done the liver was distended and rapidly blanched to a cream color. Any portion not completely blanched was discarded and the washed liver was then excised and placed in a plastic bag containing HBSS, Ca^{++}, Mg^{++} free, supplemented with 25 mM HEPES buffer, 1 mM Ca^{++} , 0.05% collagenase, 50 u/ml DNAse I and 1.5% BSA. This bag was sealed and placed in a Stomacher lab blender, an instrument which uses alternating paddles to compress the bag and help disperse the liver cells. Use of the Stomacher allows 10 livers to be processed simulataneously, and the blending action does not reduce cell viability. After blending for 3 minutes, the bag was removed and placed in a 37°C shaker incubator for 45 minutes. The resulting liver digest was filtered through 50-gauge stainless steel mesh and washed 2x with HBSS, ^{++}Ca, ^{++}Mg free 25 mM HEPES at 500 x g at 5°C. Most parenchymal cells were removed by 2 sequential centrifugations at 50 x g for 4 minutes. After a final centrifugation at 500 x g, the liver cells were resuspended in 15 ml of 30% metrizamide in Gey's balanced salt solution (4). Nine ml of HBSS, Ca^{++}, Mg^{++} free, 25 mM HEPES was then added to give a final concentration of 18% metrizamide. This suspension was then overlaid with 5 ml of the HBSS and the tubes were then centrifuged at 1400 x g for 20 minutes at 5°C. Liver nonparenchymal cells (NPC) remained at the metrizamide - HBSS interface while most of the ramining parenchymal cells, contaminating red blood cells and debris pelleted. The NPC fraction was removed, washed 3x in the HBSS and resuspended in complete EMEM. KC were isolated from the NPC fraction by selective adherence.

MØ Ectoenzyme Assays. Peritoneal cells were allowed to adhere in 35mm-diameter well plates at a concentration of 2.5 x 10^6 cells/ml/well for 2 hours at 37°C in a 5% CO_2-air mixture. Afterwards, nonadherent cells were removed by three washes with distilled water (200ul/well). The lysates were frozen at -20°C until

assayed, using 100ul for alkaline phosphodiesterase I (APD), 20 ul for 5'-nucleotidase (5'N), and 60 ul for protein determination. Protein concentration was determined using the Bio-Rad procedure (Bio-Rad Laboratories, Rockville Center, NY). 5'N specific activity (S.A.) was determined using 0.15 mM 3H-adenosine monophosphate (AMP) as the substrate and p-nitrophenyl phosphate as the competitive inhibitor of phosphatase activity (42). The APD S.A. was assessed as previously described (42) using 1.5 nM p-nitrophenyl thymidine-5'-monophosphate as the substrate. The S.A. was determined using the extinction coefficient 12000 for p-nitrophenol and was expressed as n moles of p-nitrophenol produced per milligram protein per minute at 37°C.

MØ Antitumor Assay. An adaptation of the densitometric micromethod developed by Leu and Herriott (35) was employed. Peritoneal cell suspensions were diluted to 1.2 10^6 MØ/ml and added to test wells of flat bottom microtiter plates in one of the following amounts: 200ul, 100ul, or 50ul; these corresponded to effector to target cell ratios of 20:1, 10:1 and 5:1 respectively. All wells were brought to a final volume of 200ul with complete EMEM. After 2 hours of incubation at 37°C, nonadherent cells were washed off with cold DPBS and a 200ul aliquot of LL target cells (1.2 x 10^4 cells/well) was added to the appropriate wells. To facilitate an even cell distribution, test plates were centrifuged at 55 x g for 5 minutes. Plates were then incubated for 48 hours at 37°C in a 5% CO_2-air mixture. Following incubation the supernatant fluids were decanted and the cells were washed gently with warm DPBS, fixed for 30 minutes in 33% absolute ethanol containing 1.3% formaldehyde in saline and stained with 0.5% crystal violet for one hour (61). The plates were washed, air dried and the stained monolayers were solubilized with 50% ethanol in distilled water for one hour. Tumor cell cytotoxic/cytostatic activity was determined by measuring the amount of light (570nm) transmitted through the wells using a MR 580 Microelisa Auto Reader (Dynatech, Alexandria, VA). Tumor cell destruction was calculated as previously reported (1,53). At least 3 wells/group were run and the average OD at 570 nm used.

NK Cell Assay. NK activity was assessed using a standard ^{51}Cr release assay (11). Tests were conducted in triplicate determinations in round bottom microtiter plates with varying amounts of effectors added to test wells in 100ul of complete RPMI. Target YAC-1 cells were radiolabeled with 100uCi Na_2 $^{51}CrO_4$ (New England Nuclear, Boston,MA). Approximately 1 x 10^7 cells were labeled for 2 hours at 37°C with gentle shaking every 15 minutes. Labeling was terminated by the addition of cold RPMI 1640 and the cells were then washed twice before being resuspended in complete RPMI. Labeled targets (1 x 10^4) were then added to all wells to give a final volume of 200ul/well. To facilitate cell contact, test plates were centrifuged at 55 x g for 5 minutes. Following incubation at 37°C for 4 hours, plates were centrifuged at 500 x g for 10 minutes at 5°C. A 100ul aliquot of supernatant was harvested from each well and placed in a plastic tube for counting in a gamma counter. The percent specific ^{51}Cr release was calculated as previously reported (53). To measure interferon inducible NK cell activity, mice were inoculated with 100 ug poly IC 18-24 hr prior to spleen harvest.

Intrinsic MØ Virus Interactions. Resident (Res) peritoneal MØ were obtained by our usual lavage procedures, washed and allowed to adhere for 2 hr. The adherent

Res MØ were cultured for 24 hours before infection. For infection with HSV, the number of MØ present was estimated by counting nuclei obtained after treatment of cells with cetrimide (64). The amount of virus was adjusted to provide the appropriate m.o.i., which was usually about 3 to ensure infection of all the MØ. Virus was allowed to adsorb for 1 hr, nonadsorbed virus was removed by washing, and the MØ were cultured at 37°C. For some experiments, the amount of non-adsorbed virus was estimated by titrating the amount of infectious virus in the supernatant. The amount of adsorbed but not yet eclipsed virus was estimated by titrating the amount of cell-associated infectious virus present at 1 hr. The MØ cultures were observed for cytopathic effect (CPE), and total virus yield (cell plus supernatant fluid) or supernatant virus yields were obtained and frozen until assayed by plaque titration. HSV samples were sonicated prior to titrating. Infectious virus yields were expressed as PFU per culture, and as PFU per cell in order to correct for differences in cell numbers in the cultures. Various controls were usually included in the MØ-virus experiments. A fully permissive cell line (such as Vero cells for HSV-1) was infected simultaneously with Res MØ in order to ensure the viability of the virus preparation. A thermal inactivation control, consisting of the same concentration of virus as used in the MØ infection, was incubated to establish the rate of viral inactivation in the absence of MØ.

Extrinsic MØ Virus Interactions. This procedure was similar to that previously developed by us with HSV-2 (48). For assays using HSV-1, 2.5×10^5 Vero cells were cultured overnight in 16mm tissue culture wells of 24-well plates. A sample of Vero cells was lysed with cetrimide and the nuclei counted, in order to determine the appropriate number of peritoneal cells to add to provide a ratio of ca. 1-3 MØ:Vero cell. The effector peritoneal cells were obtained by lavage from untreated mice (Res MØ) or mice treated with various immunomodulators. All immunomodulators were inoculated by a schedule identical to the prophylactic schedule (time, dose, and route) that provided antiviral protection, and the peritoneal cells were obtained on what would have been the day of i.p. challenge with HSV. By this procedure, the extrinsic antiviral activity of macrophages was assessed at an early time in infection during which macrophage antiviral activity appears to be important (47).

To perform the assay, Vero cells were infected with 100 PFU of HSV-1, virus was adsorbed for 1 hr, and nonadsorbed virus removed. Three cultures, that received no further treatment, served as controls. The appropriate number of peritoneal cells was added to the other wells containing HSV-infected Vero cells, taking into consideration differences among immunomodulator elicited peritoneal cells in regard to the proportion of cells that are MØ and the percent of MØ that adhere. After 2 hr, the nonadherent cells were removed by washing twice, and media was added. Samples containing Vero cells alone and Vero + MØ were counted, in order to calculate the actual number of effector cells present. Cultures were incubated for 48 hr before freezing cells and supernatant fluid to determine the total yield of HSV. The total PFU/culture was determined by plaque formation on Vero cells. Generally, three cultures per experimental group were used, and differences among the geometric means of the virus yields were analyzed by the paired t test. Experiments with the other viruses were performed similarly, except that different target cells were used depending upon in which cells the viruses grew optimally.

HSV-1 Viral Antigen Immunofluorescence Studies. Specific monoclonal antibodies (mab) to HSV-1 viral proteins were used on infected cell preparations to determine whether any expression of the viral proteins had occurred. Vero cells and Res MØ populations were grown and harvested at various time points following infection with HSV-1. The coverslips were then fixed in acetone for 15-20 min and air-dried. Immunofluorescence was assayed either directly, using fluoresceinated mab to VP5 (Kallestad Laboratories, Inc., Austin, TX) or indirectly, using mab to ICP4 (courtesy of L. Pereira) followed by fluoresceinated rabbit anti-mouse IgG (Miles Laboratories, Elkhart,IN). Results are expressed as the percentage of positive cells in a minimum of 300 cells counted.

Antiviral Protection Studies. B6C3F1 mice were usually randomized into experimental groups by using a computer generated random numbers series. Generally 15 mice were used in the placebo control group, and 10 mice in each experimental group. A LD_{50} titration was performed simultaneously with each experiment, in order to ensure that the appropriate number of LD_{50} doses was used for infection. A simultaneous in vitro titration of PFU/ml of the pool was also often performed. Mice were treated with the immunomodulators at the doses, routes and schedules indicated on each table of results. Infected mice were monitored daily for signs of clinical illness (ruffled fur, hunched back, paralysis, CNS symptoms) and mortality. Obviously moribund mice were sacrificed, and their day of death designated as the next day. Mice were observed usually for 21 days. The percent mortality and median survival time in days (MST) of each group were calculated.

In order to minimize data presentation, only the lowest doses that exhibited significant antiviral activity are reported. Where several experiments had placebo control groups that did not differ significantly in mortality or MST, the data for only the experimental groups are presented.

Statistical Analysis. Statistical significance for the immunomodulator data was determined by the analysis of variance with Newman-Keul's multiple-range test with the level of significance set at $p < 0.05$ (6). The mortality data were analyzed on a Apple IIe microcomputer using the Chi Square test included in the Applestat statistical package (66). This test is not overly conservative as is the Fisher's exact test or Chi Square with Yate's correction factor, and thus can point out immunoodulator regimens with moderate activity (6). The median survival time (MST) was calculated, and the survival distribution data were analyzed with the life-tables method and the Lee-Desu method of group comparison, using the SPSSX package on the VAX. This procedure allows the most appropriate analysis of survival data with censored observations (i.e. mice still alive at the end of the observation period).

RESULTS

Immunomodulator Profiles

Activation of splenic NK cells and peritoneal MØ by treatment of mice with immunomodulators. Throughout this second year of study, we completed our work on

evaluating a group of diverse immunostimulatory compounds for general modulation of nonspecific immunity (MØ activation, MK cell activity). We have a manuscript in press summarizing these results. The two final experiments done in this study year were dose response analyses of the effect of treatment of mice with Ampligen on MØ ectoenzyme specific activities (Table 3) and of treatment with CL246,738 on MØ ectoenzyme specific activities and antitumor activities (Table 4). Both compounds caused some changes in peritoneal macrophages as evidenced by reductions in the 5'N ectoenzyme activity; we have previously noted the exquisite sensitivity of this marker to any changes in the peritoneal compartment (1,42). The experiments also confirmed that these two immunomodulators induced moderate MØ activation as assessed by signficant, but modest descreases in the APD activity, as compared with the marked decrease in C. parvum MØ. This parallelled the moderate degree of MØ antitumor activity observed for CL246,738 (Table 4) or Ampligen (Annual Report, December 31, 1986).

Broad spectrum intrinsic antiviral resistance of peritoneal Res MØ. We completed our studies this year that demonstrate that freshly harvested Res peritoneal MØ are relatively nonpermissive for a broad spectrum of viruses, as evidenced by none or very low production of infectious virions and no cytopathic effect (Table 5). Some viral gene expression does occur in Res MØ infected with HSV-1 or HSV-2 (manuscript submitted), and it is possible that some gene expression may occur with the RNA viruses as well. Although intriguing, that experimental question is outside the contract workscope.

Intrinsic antiviral activity of immunomodulator activated MØ . We also evaluated whether immunomodulator activated MØ exhibited enhanced intrinsic antiviral activity to HSV-1 or Pichinde virus (Table 6), or any differences in response to virus infection, as compared with Res MØ. Res and immunomodulator activated MØ exhibited marked intrinsic antiviral resistance to HSV and Pichinde virus. All of the MØ were extremely resistant to virus infection, as evidenced by a marked decline in infectious PFU per cell. Although the data suggested that immunomodulator activated MØ exhibited increased intrinsic antiviral resistance to HSV-1, there were no significant differences in the degree of antiviral resistance between Res MØ or immunomodulator activated MØ when the data were analyzed by ANOVA using the Tukey posthoc t test.

None of the MØ tested showed significant cytopathic effect after HSV infection, except for C. parvum activated MØ which showed considerable cytopathic effect (manuscript submitted). Although none of the MØ showed cytopathic effect after infection with Pichinde virus, infection of Res, CL246,738 activated, or other immunomodulator activated MØ (data not shown) with Pichinde virus caused a definite increase in the spreading and a probable increase in the number of cells present. Cell counts from photomicrographs indicated that Pichinde virus infected Res MØ increased 2 fold in number, while CL246,378 MØ increased 1.8 fold. We have demonstrated that live virus is required for the effect, because UV inactivated Pichinde virus was without effect (data not shown). The results do not appear to be the result of mycoplasma contamination, because our Vero cells were recently checked by Bionique and found to be negative. Although not in the contract workscope, as time permits we will investigate the mechanism responsible for this novel interaction of MØ with Pichinde virus.

Extrinsic antiviral activity of Res and immunomodulator activated peritoneal MØ. The ability of various MØ to inhibit HSV, Pichinde, Banzi and Semliki forvest virus replication in normally permissive cells was evaluated. Res MØ exhibited moderate extrinsic antiviral activity for HSV replication in Vero cells (Table 7). Most of the MØ obtained from mice treated with immunomodulators also showed significant extrinsic activity, and inspection of the data suggests that the activity is greater than that exhibited by Res MØ. More data need to be accumulated, however, before we can conclude definitively whether any of the immunomodulator activated MØ exhibit a significant increase in extrinsic antiviral activity as compared with the Res MØ. C. parvum activated MØ showed an average reduction in HSV-1 titer of 1.67 $\log_{10}$. This activity, however, appeared to be associated with cytotoxic activity of these MØ for HSV infected Vero cells. Res MØ also showed significant extrinsic antiviral activity against Pichinde virus infection in permissive Vero cells (Table 8). MØ populations, however, activated by treatment of mice with immunomodulators showed an significant enhanced extrinsic antiviral activity.

Summary of immunomodulator profile data. A summary of our findings in regard to immunomodulation of nonspecific immune responses by antivirally active compounds is shown in Table 9; the data come, from the current report, our previous Annual Report (31 December 1986), our manuscript in press (53) and other published data (46). The activities were measured on what would have been the first day of viral infection after prophylactic treatment with the compounds. It is apparent that most of the immunomodulators that are tested by a prophylactic regimen activate NK cell cytotoxicity and activate macrophages to at least some degree. However, it is also apparent that no single nonspecific immune parameter that was measured can be correlated consistently with antiviral activity. For example, MVE-2 and C. parvum are among the most active compounds in regard to in vivo antiviral activity, yet they induce none to very low levels of IFN activity or NK cell cytotoxicity by the treatment schedule used (5,10,39,41). Likewise, the variations in degree of peritoneal macrophage activation do not appear to correlate with differences in degree of antiviral activity.

These results have suggested to us that: (i) there may be no unifying mechanism of antiviral action. Each immunomodulator, tested has pleiotropic effects on nonspecific immune parameters. Protection against each virus infection may involve different aspects of host resistance, an increase in any one of which may produce increased resistance. (ii) Nonspecific immune activities other than the ones measured may be involved in antiviral protection. We may not yet have identified the unifying immune parameter. For example, the ability for an immunomodulator to activate peritoneal cells for antitumor activity may not be central to its antiviral activity. We will address this issue by determining the effects of depletion of various cell types on antiviral resistance and effectiveness of the immunomodulators. (iii) Antiviral activity of cells other than those investigated may be primary in antiviral resistance. For example, liver Kupffer MØ may be the most relevant cell in regard to antiviral activity, especially for infection with Caraparu virus which targets to the liver.

Isolation and characterzation of Kupffer cells (KC). During the contract year we

have finalized our isolation procedure for Kupffer cells (see experimental methods) and basic characterization (Table 10). We are recovering about 1.1 to 7.0 x 10^6 NPC/mouse with a total KC yield of about 0.2 to 1.0 x 10^6 KC/mouse. These results are similar to those reported by other researchers using similar isolation procedures (34,72,73).

To further purify KC we have used selective adherence to untreated plastic surfaces. KC purity, as judged by phagocytosis of opsonized SRBC, has indicated that about 65% of the 24 hour adherent cells are phagocytic and thus can be classed as KC. Most of the remaining cells have a small elongated morphology, consistent with liver endothelial cells (LEC). The possible presence of LEC has presented a problem to us because LEC may affect certain parameters (such as ectoenzyme phenotype, and intrinsic and extrinsic antiviral activity) where we are analyzing activity of the population as a whole rather than activity on an individual cell basis.

For this reason, we have proceeded with much more caution than others have studies where KC purity is not so essential. For example, one cannot report pure KC populations on the basis of: (i) positive stain for nonspecific esterase, (ii) possession of an Fc receptor for IgG, and (iii) nonspecific phagocytosis of particles such as latex beads, because LEC also stain positively for esterase, possess Fc receptors and are capable of nonspecific phagocytosis (51). In our work, we have used more specific markers to assess KC percentages (Fc mediated phagocytosis). We have, however, questioned the true identify of the non phagocytic cells in our culture because endothelial cells are reported to stick poorly to untreated surfaces and are often difficult to maintain in culture (personal communication, Dr. Kefalides, University of Pensylvania School of Medicine). In addition, in two experiments measuring the intrinsic resistance of resident KC against HSV-2, we found no CPE and no virus replication. Similar to Res peritoneal MØ, Res KMO expressed a high degree of antiviral resistance. The estimated input m.o.i. of 3 PFU/cell was reduced to about 0.05 PFU/cell in 24 hr, and to 0.025 PFU/cell by 48hr, with no apparent virus induced CPE. These data are inconsistent with the presence of LEC because endothelial cells are reported to be highly susceptible to HSV (37).

Consequentely, we have spent a great deal of time assessing LEC contamination in our NPC fraction. We have approached this problem from three different standpoints. First, we have attempted to monitor LEC numbers using LEC specific markers. Second, we have attempted to monitor KC numbers using KC specific markers. Third, we have used flow cytometry in many of these studies to help characterize positive staining cells and we have analyzed the light scatter of the NPC and of the 2 hour and 24 hour nonadherent fractions.

LEC Studies: We have looked at the LEC levels in our preparations using three reportedly endothelial cell specific markers. These are: 1) specific binding to the lectin Bandeiraea simplicifolia (BS), 2) production of the coagulation factor VIII, and 3) uptake of ovalbumin.

Specific binding to BS: Specific binding of BS has also been used as a marker for brain endothelial cells (personal communicaton, Dr. Jospeh, Thomas Jefferson University). Therefore, we attempted to use fluoresceinated BS as an index of LEC contamination in our preparations. Our results (data not shown)

indicated that this lectin was bound by both LEC and KC, and thus was not a useful LEC marker, in our system.

Production of the coagulation factor VIII: LEC have been reported to synthesize and release the coagulation factor VIII (38). To assess whether the LEC were positive for factor VIII, NPC were fixed overnight in 70% ethanol, washed, and then incubated with rabbit anti human factor VIII (Accurate Chemicals) on ice for 30 minutes. This antibody reportedly reacts with mouse brain endothelial cells (personal communication, Dr. Gary Bradshaw, University of Medicine & Dentistry of New Jersey). NPC were then washed and incubated with a second antibody (goat anti rabbit IgG-fluorescein conjugated) on ice for 30 minutes. The cells were examined for fluorescence in a fluorescent microscope. The results (data not shown) indicated no positive cells, suggesting either that the LEC were negative for factor VIII or that the anti human factor VIII antibody did not cross react to the mouse LEC (we were unable to find anti-mouse factor VIII). Nevertheless, like the leccin BS, factor VIII could not be used as a LEC marker in our system.

Uptake of ovalbumin: Uptake of fluoresceinated ovalbumin (FOA) has been used as specific marker of rat LEC (51,69). In a preliminary experiment, we stained the 2 hour and the 24 hour adherent NPC population for uptake of FOA. At 2 hours, 60% of the adherent cells were positive for FOA. Most of the positive cells had the characteristic LEC morphology (small elongated cells). At 24 hours, only 2% of the adherent cells were positive for FOA. This supports our previous finding (15 January 1987, 15 April 1987 quarterly reports) that at 24 hours most of the LEC detach from the culture substrate. At 24 hours, however, there were still LEC like cells present. These cells, which constitute about 30% of the 24 hour adherent population are nonphagocytic, and do not appear to be small KC. Their failure to take up FOA suggests that they are dead or dying LEC. To help answer this question, we will stain the 24 hour adherent cultures with propidium iodide, (62), a sensitive index of cell viability.

We also analyzed the FOA staining pattern of liver NPC on a flow cytometer. Figure 1a, and to gure 1a shows the flow cytometry analysis of the whole NPC fraction, the X axis being green fluorescence and the Y axis indicates log right angle light scatter (LRALS). Cells falling to the left of the verticle gate (69% of NPC) are negative for uptake of FOA, while cells to the right of the gate are positive.

The rest of Figure 1 shows cell number plotted against FOA fluorescence intensity in one demensional histograms. At 2 hours (figure 1b) 78% of the nonadherent (NA) population were cells positive for FOA. Similarly, the 24 hour NA population was 89% positive for FOA. Collectively, these data indicate that by 24 hours most of the LEC are no longer adherent. In support of this, there was a 12 fold decrease in fluorescence intensity when the mean log fluorescence intensity (MLFI) of the summed 2 and 24 hour NA populations (Figure 1c) was compared with the MLFI of the 24 hour adherent population (Figure 1d), (Figure 1e). Collectively, these data support the above findings that KC are negative for FOA, and that by 24 hours the remaining adherent LEC take up little FOA and may represent dead or dying cells.

KC studies: We examined our NPC for F4/80 and MAC-1 positive cells, and for

the incidence of cells bearing Fc receptors for the different classes of IgG.

F4/80 and MAC-1: Using flow cytometry, we did not find any NPC that were positive for F4/80 or MAC-1. The abscence of MAC-1 positive cells in our cultures was not surprising because KC are reported to be MAC-1 negative (34). The negative results with the F4/80, however, are surprising because F4/80 is considered a pan marker for macrophages (50), and KC have been reported to be positive (34). Our failure to find F4/80 positive cells may relate to the method we used for detection. We used indirect immunofluorescence with a phycoerythrin (PE) labeled second antibody. We used the PE label because it is red and we were trying to assess whether there were cells that would double label with fluoresceinated BS and F4/80. The PE second antibody was only available as a whole IgG molecule, consequently we found a high level of nonspecific binding to Fc receptors. This, coupled with the finding that KC showed a large amount of autofluorescence at the frequency at which PE is read, made it difficult to pinpoint any F4/80 positive staining cells. Therefore, in our future studies with F4/80 we plan to use a Fab fragment fluoresceinated second antibody.

Fc receptor studies: KC possess Fc receptors for IgG2a and IgG2b, whereas it has been reported that LEC possess Fc receptors for IgG2b only (34). To determine whether this difference could be used to assess KC purity, we performed the following experiment. NPC were incubated with Fc fragments from the various classes of IgG. To measure binding, a fluoresceinated second antibody was added and the NPC were then analyzed for fluorescence intensity on a flow cytometer. The results (Figure 2) are shown as number of cells vs. fluorescence intensity plotted in one demensional histograms. Both the minumun (left column of histograms) and maximun (right column of histograms) percent positive cells (those beyond the vertical gate) are shown. The minimun percent positive gate was set to enclose an area containing 99% of the cells stained with the FITC goat anti-mouse control antibody. The maximun percent positive cells was determined by simply subtracting out the control cells from each test population. As shown in figure 2, there were from 3-32% positive cells for IgG1, 21-69% for IgG2a, 11-65% for IgG2b, and 1-7% for IgG3. When the minumun percent positive IgG2a population was sorted, the recovered cells consisted of about 70% KC, with most of the remaining cells being lymphocytes. This represents a three-fold increase in the proportion of KC over that which we usually recover. These preliminary data indicate that the IgG2a Fc receptor marker may be a good index of KC purity in adherent populations. We therefore plan to contune our studies with this marker.

Flow cytometry analysis of peritoneal cells (PC) and liver NPC): In order to characterize the light scatter profile of liver NPC and of the NA populations, these various fractions were compared with the light scatter of the well characterized PC.

One parameter analysis: The LRALS of the 2 hour and the 24 hour NA PC and NPC are shown in figures 3 and 4, respectively, as one demensional histograms. For both PC and NPC the LRALS profile of the 2 hour and 24 hour NA fractions were summed, and then subtracted from the total input population to yield the LRALS of the 24 hour adherent population. The gates in which the 24 hour adherent cells fell were projected onto a 2 parameter histogram (bottom of figures 3 and 4), which enabled them to be located in the light scatter profile of the whole NPC population.

Two parameter analysis: In the 2 parameter analysis, the forward angle light scatter (FALS) was plotted against the LRALS for the input cell population and for the 2 hour and 24 hour NA fractions. The areas in which the various cell populations fell were mapped, and the number of cells falling in each map was quantitated for each analysis. As shown in figure 5, there were 3 distinct populations of PC: a left population of lymphocytes, a right population of macrophages, and a center population consisting probably of macrophages and large lymphocytes. In contrast, it was difficult to map three distinct populations of NPC (Figure 6). This indicates how heterogeneous the NPC are as compared with PC, and points out that these cells will be more difficult to characterize by this type of analysis.

Ectoenzyme profiles of Kupffer cells. Using the purity level achievable by adherence, in two preliminary experiments we investigated the effect of rMuIFN-G, MVE-2, and C. parvum on the KC ectoenzyme profile. The data for rMuIFN-G (Table 11) include as a comparison the prototype peritoneal MØ from control and rMuIFN-G treated mice. More data need to be obtained before we can establish firmly the ectoenzyme activities. However, our current impressions are that treatment of mice with rMuIFN-G altered the ectoenzyme profile of KMØ as well as peritoneal MØ to that of an antitumor MØ, with significant reductions in both 5'N and APD ectoenzyme specific activities. The APD levels for Res KMØ varied considerably in the two experiments performed. It is notable, however, that the activity was 5-10 fold greater than that of Res peritoneal MØ (Table 12). Whether the high APD activity is characteristic of KMØ or is due to high APD activity in contaminating endothelial cells, and whether the variability is related to differing contamination in different experiments, will be addressed in future studies. It should be noted that we have found that Res peritoneal MØ from rats are also quite high in APD activity (40).

Selective Depletion of Tissue MØ and NK Cells

Three novel experimental procedures for selective depletion were evaluated during this contract year. The most promising appears to be local depletion of either peritoneal MØ or of splenic and Kupffer MØ with toxins encapsulated within liposomes.

Effect of Mab to NK1.1 antigen on NK cell activity in the B6C3F1 mouse. Several experiments were performed with different doses and schedules of treatment of adult mice with the IgG2a Mab NK1.1, which has been used quite successfully to deplete neonatal mice of NK cells (32). Its effects on antimicrobial host resistance, however, have not been evaluated. We demonstrated that single i.p. treatment of a low NK responsive mouse, the CD-1 mouse, three days prior to assay effectively reduced IFN inducible splenic NK cell activity to 14% of the control level. However, this regimen was relatively ineffective in reducing either spontaneous or polyI:C (IFN) inducible NK cell activity in the adult B6C3F1 mouse, even when the dose was increased to 200ul (Table 13). In an attempt to increase efficacy, mice were pretreated with the IFN inducer, poly I.C, prior to

treatment with Mab NK1.1 on the hypothesis that this might increase the number of target antigens. However, this regimen failed to increase the efficacy of Mab NK1.1 in adult B6C3F1 mice (Table 14). Finally, mice were treated repetitively with Mab NK1.1 every 10 days; this regimen was no more effective than was single treatment (Table 15). It is apparent that this Mab is relatively ineffective in the adult B6C3F1 mouse, especially against IFN enhanced NK cell activity. For this approach to be useful either a different mouse strain will have to be used, or treatment initiated in neonatal life and continued weekly thereafter. Effort on this approach will be minimized until other depletion methods that are effective have been fully characterized.

Effect of i.v. inoculation of the toxin DMDP encapsulated in liposomes on MØ, NK cells and host resistance. The toxin dichloromethylene diphosphonate (DMDP) has profound depleting effects on splenic and liver MØ (68) and humoral immune functions in these organs (12); however, its effects on host resistance have not been determined. The DMDP depletion system is particularly interesting , because it is reported to selectively deplete for about one week splenic and liver MØ, two of the most important tissue MØ populations without affecting other tissue MØ compartments. During this year, we have characterized the effects of this toxin in the CD-1 mouse, in preparation for subsequent work in the B6C3F1 mouse.

We established that two i.v. injections of DMDP liposomes (days -4 and -2 prior to assay) caused a marked decrease in splenic NK cell activity and a marked leukocytosis of lymphocytes and PMN (Table 16), but no significant effect on peritoneal cell populations or on peritoneal MØ ectoenzyme profiles (Table 17). Double treatment was necessary, because single treatment did not affect circulating leukocytes or splenic NK cell levels (Table 18) and did not decrease resistance of CD-1 mice inoculated i.v. or i.p. with HSV-2 (Table 19). The study of the kinetics of the effect of double i.v. treatment with DMDP liposomes revealed that the leukocytosis of the lymphocyte and PMN populations persisted for at least 6 days, although the populations, were returning toward normal by day 6 (Table 16, Fig. 7). Interestingly, there was no pronounced monocytosis. We speculate that the leukocytosis is related to an outflow of the large circulating pool of lymphocytes and PMN that is contained within the spleen and liver, due to destruction of the tissue macrophages and subsequent alterations in organ architecture. The fact that the increase in circulating leukocytes occurred so rapidly, and the fact that the cells mostly appeared to be mature, argues against the leukocytosis being due to enhanced maturation of bone marrow stem cells. Cytochemistry on frozen spleen sections for the presence of acid phosphatase and the F4/80 MØ antigen confirmed the loss of splenic MØ after double i.v. treatment (van Rooijen, unpublished observations).

The data point out the complexity of any of the available depletion methods for MØ, because splenic NK cells were depressed as well as splenic MØ. Thus, it is necessary to characterize completely the depletion system in order to interpret changes found in host resistance. The effect on NK cells appears to be short lived, with normal cytotoxicity being measured by 4 days after DMDP liposome treatment (Table 20). Only on the first one or two days after administration of DMDP liposomes were the spontaneous and interferon inducible NK cell activity significantly reduced from normal levels (Tables 16,18,20).

The data showing no change in the peritoneal Res MØ population (Table 16,17

and Fig. 7) after i.v. administration of DMDP in multilamellar liposomes, which cannot penetrate capillaries and gain entrance into the peritoneum, provide additional support for the concept that peritoneal MØ are separated from at least several other MØ compartments in the body (55). There was a slight decrease in natural resistance of mice that were treated twice i.v. with DMDP liposomes and infected i.p. with HSV-1 when the overall data were analyzed by Cox's proportional hazards general linear model (Table 19). However, this slight decrease in resistance to infection initiated by the i.p. route was marginal compared with the marked (more than 100 fold) decrease in resistance to i.v. infection with either HSV-2 (Table 19) or Listeria monocytogenes (Table 21). The question now to be addressed is whether such depletion of splenic and liver MØ and decrease in natural resistance can be overcome by treatment of mice with immunomdulators.

Effect of i.p. treatment of mice with ricin encapsulated in liposomes. As a counterpoint to the i.v. toxin liposome experiments, CD-1 mice were inoculated i.p. with ricin liposomes (collaboration with Dr. Alvin Volkman, East Carolina University School of Medicine). The toxin was inoculated into normal CD-1 mice and into mice that had been treated with 89Sr to deplete the mice of monocytes and any inflammatory exudate of monocyte-MØ to the peritoneal cavity after ricin liposome treatment (58). The four groups of mice were then infected i.p., 24hr after ricin liposome treatment, with Listeria monocytogenes. Treatment of either control or 89Sr treated mice with ricin liposomes caused a marked reduction in resistance to Listeria (Table 22).

Thus, we now appear to have two distinctly different methods with which to selectively deplete mice of either the splenic and liver MØ populations, or the peritoneal MØ population, without affecting the other compartment. The lack of effect on the respective tissue MØ compartments will be confirmed by isolation of Kupffer and peritoneal MØ.

Broad Spectrum Antiviral Efficacy of Treatment of Mice with Immunomodulators

During this second contract year, we have continued to evaluate the antiviral efficacy of a variety of immunomodulators against herpesvirus, flavivirus, alphavirus and bunyavirus infections.

Antiviral efficacy of immunomodulators against Banzi flavivirus. Intraperitoneal infection of mice with Banzi virus continued to provide a very sensitive model infection in which to evaluate immunomodulators. The results of several experiments are summarized in Table 23.

A variety of therapeutic schedules were tested. Delaying treatment with CL246,738 until 24 hr after infection removed almost all antiviral activity. In contrast with CL246,738, repeated therapeutic treatment with Ampligen, beginning 24 hr after infection, provided significant antiviral activity against Banzi virus in two experiments. Moreover, treatment with Ampligen that was delayed until 48 hr after infection still showed partial activity. Similar repeated therapeutic treatment with rHuIFN-A A/D beginning 24 hr after infection, was also

partially effective in two experiments. The slight variations in efficacy noted between experiments do not appear to be related to differences in the Banzi virus challenge dose and severity of infection. The variation may be related to the fact that the therapeutic regimens are not optimum (as compared with prophylactic treatment). These results emphasize the need to confirm efficacy in completely independent experiments. The differing therapeutic efficacies with rHuIFN-A A/D and the IFN inducers CL246,738 and Ampligen suggest that IFN induction may not be the only mechanism of antiviral activity of these compounds, or that other differences such as pharmacokinetics may be responsible.

Several new immunomodulators were evaluated against Banzi virus. Prophylactic treatment with the pyrimidinones was generally effective. Combined repeated prophylactic and therapeutic treatment with the recombinant DNA derived biologicals (rHuCSF-M or rHuTNF-A) was completely ineffective.

Antiviral efficacy of immunomodulators against Semliki forest virus infection. Parenteral infection with this alpha togavirus also provided a very sensitive infection model in which to evaluate immunomodulators (Table 24). Prophylactic treatment with either of the two positive controls, MVE-2 or C. parvum, provided complete protection. Single prophylactic administration of CL246,738 or the three pyrmidinones was also very effective. As has been shown with Banzi virus, CL246,738 was completely effective at a dose as low as 30 mg/kg. It remains to be determined whether therapeutic administration of CL246,738 will be effective against the alpha togavirus. Treatment with Ampligen was quite effective in either a single prophylactic regimen or a repeated therapeutic regimen beginning 4 hr after infection. Investigation will now be focused on establishing whether treatment can be delayed until 24 to 48 hr, as has been shown to be effective against Banzi virus.

In regard to treatment with the IFNs, rHuIFN-A A/D was more effective than was rMuIFN-G, as we have previously noted (46,53). rHuIFN-A A/D provided excellent protection when administered in a single therapeutic dose 4 hr after infection, or in a repeated regimen beginning 24 hr after infection. rMuIFN-G, in contrast, was effective when administered in two doses 24 hr before and right after infection, but was only marginally effective with the single dose given right after infection. Repeated daily treatment with rMuIFN-G beginning 2 hr after infection was effective, but unlike rHuIFN-A A/D, treatment could not be delayed until 24 hr after infection.

In our single experiment with repeated combined prophylactic/therapeutic treatment with rHuCSF-M, no antiviral efficacy was observed. Repeated combined prophylactic and therapeutic administration of the bacterial cell wall preparations, MPL + TDM + CWS, provided excellent antiviral activity.

Antiviral efficacy of immunomodulators against Caraparu bunyavirus infection. As we have noted previously (53), infection with Caraparu was the most insensitive to treatment with immunomodulators (Table 25). Prophylactic treatment with the positive control, MVE-2, provided significant, but not the complete protection found with the other viruses. Likewise, single prophylactic administration of CL246,738 required an increase to a dose of 400 mg/kg for protection to be evident. Moreover, repeated early therapeutic treatment with CL246,738 provided only modest protection, and was associated with drug toxicity. The degree of

antiviral efficacy was closely related to the dose of challenge virus. When the challenge produced 63% mortality, the CL246,738 treatment group showed both a significant delay in death and a decrease in mortality, but when the challenge dose was increased there was only an increase in the MST.

A regimen of prophylactic treatment with Amligen combined with one dose shortly after Caraparu virus infection was ineffective, even at a dose of 8 mg/kg. Repeated combined prophylactic and/or therapeutic administration at 4 mg/kg provided variable protection, depending on the challenge dose.

The results with treatment with the two recombinant IFNs comfirmed the relative insensitivity of Caraparu virus to IFN or IFN inducers. Repeated combined prophylactic and/or therapeutic administration of rMuIFN-G only provided partial protection. Repeated combined prophylactic and/or therapeutic administration of rHuIFN-A A/D appeared to be less effective than the rMuIFN-G, in contrast with the results with Banzi and Semliki forest virus. These apparent differences in antiviral efficacy of rHuIFN-A A/D and rMuIFN-G merit further investigation.

The single experiments that have been performed with rHuIL-1 B and rHuTNF-A revealed no marked antiviral activity when these drugs were administered on a repeated combined prophylactic and therapeutic regimen. Prophylactic treatment with the pyrimidinones likewise was ineffective.

Antiviral efficacy of immunomodulators against HSV-2 infection. In this contract year, we have focused on establishing whether treatment with antivirally active immunomodulators could be shortened or delayed (Table 26). As with the other infection models, prophylactic treatment with either of the two positive control immunomodulators, MVE-2 or C. parvum, provided complete protection. Single prophylactic treatment with two of the pyrmidinones was effective. Ampligen was shown to be quite effective upon repeated therapeutic administration beginning 4 hr after infection; whether treatment can be further delayed is currently being invesigated.

Investigation of the various regimens for treatment with rHuIFN-A A/D revealed that antiviral efficacy was still maintained if drug treatment was delayed until 24 hr after infection, and that only two administrations of drug are necessary. These results are very encouraging for use of rHuIFN-A A/D as an antiviral compound. As we have previously observed, rMuIFN-G was less effective, because single prophylactic treatment provided only partial protection, and single early therapeutic treatment 4 hr after virus infection was not effective. However, repeated therapeutic treatment with rMuIFN-C was quire effective against HSV-2 infection if it was begun by 4 hr after infection.

Our results with repeated combined prophylatic/therapeutic treatment with rHuIL-1 B were inconsistent. In the first experiment, excellent antiviral protection was observed, but this was not repeated in the validation experiment. Our stock is being retitered to determine whether a loss in antiviral protency could be attributed to loss of compound activity. As with the other virus infections, single experiments testing treatment of mice with rHuTNF-A or rHuCSF-M did not provide marked antiviral activity against HSV-2 infection.

Antiviral efficacy of ribavirin against alpha-, flavi-, and bunyavirus infections. In contract with our immumodulator data, the nucleoside analogue ribavirin was most effective against Caraparu bunyavirus, moderately effective against Banzi flavi virus and ineffective against Semliki Forest alphavirus (Table 27). When administered in either a combined repeated prophylactic/ therapeutic or a repeated therapeutic treatment regimen, ribavirin provided complete protection against systemic challenge with Caraparu. Similar treatment against Banzi significantly reduced mortality from 100% in control mice to 60% in ribavirin treated mice. Results with Semliki Forest virus, the most sensitive model in our immunomodulator studies, indicated this infection was resistant to the antiviral activity of ribavirin.

DISCUSSION

Comparative analysis of the antiviral effectiveness of immunomodulators or the chemotherapeutic drug ribavirin has continued to emphasize broad spectrum antiviral activity against herpes simplex virus and the alphatogavirus, flavivirus and bunyavirus infection models tested (46,53). The active immunomodulators were most effective against Semliki forest alphavirus and Banzi flavivirus infections, and were least effective against Caraparu virus infection which was by far the most insensitive to the antiviral efficacy of immunomodulators. In contrast with the immunomodulators, ribavirin treatment was very effective against the Caraparu bunyavirus infection, much less so against the flavivirus infection, and ineffective against the alphavirus infection. These results on the antiviral spectrum of ribavirin are consistent with the data of others, indicating the greatest effects on bunyaviruses and arenaviruses (7,21).

The difference in sensitivity to immunomodulators between Caraparu virus and the other viruses such as Banzi flavivirus and Semliki forest and Venuezuelan equine encephalitis alphaviruses is intriguing. Whether the differences are related primarily to the different replication strategies of the viruses, or to the different pathogenesis patterns reamins to be determined. Caraparu virus appears to replicate preferentially in the liver and causes death through hepatic invovlement (Shope, unpublished observations), while death from Banzi and SFV infections comes from the effects of infection in the central nervous system. In this context, ribavirin is reportedly active against viral hepatitis (57) and this may explain why Caraparu was most sensitive to ribavirin treatment.

Comparison of prophylactic and therapeutic administration of active immunomodulators has continued to emphasize the fact that immunomodulators are most effective when administered prophylactically or close to the time of infection (46). A few immunomodulators, however, were effective when administered on a therapeutic regimen. The most notable examples were the protective effects of Ampligen, rMuIFN-G and rHuIFN-A A/D.

Whether antivirally active MØ are a common pathway for the antiviral activity of the active immunomodulators has yet to be determined. We found that resident peritoneal MØ exhibited marked intrinsic antiviral activity in vitro

against all of the viruses tested, emphasizing the general non-permissiveness of MØ for virus infection (15,33,44,59,67). All of the immunomodulator activated peritoneal MØ that were tested also exhibited marked intrinsic antiviral activity to HSV-1 and Pichinde arenavirus infections.

We have previously demonstrated that certain immunomodulator activated peritoneal MØ, as compared with resident peritoneal MØ, expressed enhanced extrinsic antiviral activity to HSV-2 replicating in permissive cells (48). In the present study, we found that Res MØ, as well as most immunomodulator activated MØ, significantly reduced the yields of HSV-1 in permissive Vero cells. As we have shown with HSV-2, activated MØ did exhibit increased extrinsic activity to Pichinde virus as compared with the Res MØ. It will be of interest to determine whether activated MØ also express enhanced extrinsic antiviral activity to alpha- and flavi-viruses. In general, our data indicate that resident and immunomodulator activated peritoneal MØ have a high degree of both intrinsic and extrinsic antiviral activity against diverse viruses. The antiviral ability of liver Kupffer MØ, an important organ in viral pathogenesis, will be the focus of studies for the following year.

While establishing the antiviral activity of MØ in vitro can indicate the possibility that MØ may be involved in the antiviral activity of immunomodulators, defining the effects on infection of selective depletion of MØ provides an important and complementary independent approach to establishing the role of MØ. We have established that treatment of CD-1 mice with the toxin, DMDP-L, causes a profound 100-fold or greater decrease in resistance of mice to i.v. infection with HSV-2. Whether the immunosuppression can be produced similarly in B6C3F1 mice, or reversed by treatment of mice with immunomodulators remains to be determined. The treatment appears to act locally and selectively on splenic and liver cells, (this report), rather than systemically, because peritoneal cell populations were not affected. The rapid effects on the peripheral circulating leukocytes also argues for a local disruption of the architecture of the spleen and/or liver leading to a release of organ associated lymphocytes and marginated granulocytes. In contrast, there was not a marked effect on circulating monocytes, which may argue against the existence of a large pool of marginated monocytes located within the spleen or liver.

Another method to deplete mice selectively of effector cells, NK cells, was also evaluated, but found not to be useful for B6C3F1 mice. Treatment of B6C3F1 mice with various regimens of the monoclonal antibody, Mab NK 1.1, failed to reduce sufficiently either spontaneously or interferon inducible splenic NK cell activity. This is in contrast to the marked depleting effects of this reagent on NK cell activity in the CD-1 mouse (this report) or in other mouse strains (32). The difficulty may lie in such factors as (i) the high spontaneous NK cell activity that is present in the B6C3F1 mouse, as compared with the CD-1 mouse, (ii) the possible lack of sufficent receptors for Mab NK 1.1 on the surface of cells in the B6C3F1 hybrid mouse as compared with the parental C57Bl/6 mouse, or (iii) in other factors such as possible earlier maturation of NK cells in the B6C3F1 mouse.

The results with evaluation of methods for selective depletion of effector NK cells or MØ emphasizes the complexity and interrelationships that exist between these two cell populations. It is apparent that considerable systematic

characterization needs to be performed before results with studies using such depletion methods can be interpreted clearly. Nevertheless, the combination of selective depletion methodology, coupled with correlative studies of the antiviral activity of effector MØ or NK cells, are powerful experimental tools for establishing whether the antiviral activity of immunomodulators involves or requires MØ or NK cell antiviral activity.

PUBLICATIONS RESULTING FROM THIS WORK

1. Morahan, P.S., E.R. Leake, D.J. Tenney and M.S. Sit. Comparative analysis of modulators on nonspecific resistance against microbial infections. In: Immunopharmacology of Infectious Diseases: Vaccine Adjuvants and Modulators of Nonspecific Resistance (Ed. J. Majde), Alan R. Liss, Inc., New York, NY, pages 313-324, 1987.

2. Pinto, A.J., P.S. Morahan, and M. Brinton. Comparative study of immunomodulatory and antiviral activity of various immunomodulators. Int. J. Immunopharm., in press, 1988.

3. Morahan, P.S., D. Stewart, and A.J. Pinto. Role of macrophages in viral infections. Abstract, UCLA Symposium on Liposomes in the Therapy of Infectious Diseases and Cancer, February 1988.

4. Pinto, A.J., D. Stewart, P.S. Morahan and M. Brinton. Comparison of antiviral efficacy and mechanism of action of immunomodulators against exotic RNA viruses. Abstract, International Conference on Antiviral Research, April, 1988.

STATEMENT OF PLANS FOR THE NEXT YEAR

During the third year of the current contract, we will continue to evaluate a few carefully selected new compounds for broad spectrum antiviral activity against alphatogavirus, flavivirus, bunyavirus and herpes simplex virus infections. We plan to concentrate, however, on therapeutic treatment and combination treatment with different immunomodulators known to be effective or with immunomodulators in combination with ribavirin. We will continue to investigate the intrinsic and extrinsic antiviral activity of peritoneal MØ, and will compare these activities with those of liver Kupffer MØ. Finally, we will evaluate the effects of selective depletion of splenic and liver cells by DMDP-liposomes or of monocytes with ^{89}Sr on natural and immunomodulator induced antiviral resistance.

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Table 1. Immunomodulators

Name	Composition	Source	AVS Number
Microbials			
C. parvum	Killed bacterial vaccine	Burroughs Wellcome	-
TDM	Trehalose dimycolate	Ribi Chem	2155
MPL	Detoxified monophosphoryl lipid A	Ribi Chem	2153
CWS	Cell wall skeleton from Mycobacterium	Ribi Chem	2154
PA-PE	Pyridine extract from C. parvum	Ribi Chem	2157
Synthetics			
CL246,738	3,6-Bis(2-piperidinoethoxy) acridine	Lederle	-
ABPP	2-NH2-5-Br-6-phenyl-4(3H)-pyrimidinone	Upjohn	2776
AIPP	2-NH2-5-I-6-phenyl-4(3H)-pyrimidinone	Upjohn	2777
ABMP	2-NH2-5-Br-6-methyl-4-(3H)-pyrimidinone	Upjohn	2778
MVE-2	Maleic anhydride divinyl ether copolymer	Hercules	-
Ampligen	Mismatched poly rI:rC	Johns Hopkins	2149
GE-132	2-carboxyethylgermanium sesquioxide		3934
HDPP	DL-2,3,5,6,7,8-hexahydro-8,8-Dimethoxy -2-phenylimidazo(1,2,-A)pyridine Hydrochloride	-	2880
S-209	Analoge of peptidoglycan	Ribi Chem	2158
Recombinant DNA derived compounds			
rMuIFN-G	Murine gamma interferon	Genentech	2351
rHuIFN-A A/D	Human alpha A/D interferon	Hoffman LaRoche	-
rHuIL-1 B	Human beta interleukin 1	Smith Kline French	-
rHuTNF-A	Human tumor necrosis factor alpha	Genentech	-
rHuCSF-M	Human macrophage colony stimulating factor	Cetus	2269

Table 2. Virus Strains

Virus Group	Strain	Source of Pool or Seed	Virus Titers		
			In vitro PFU/ml	In vivo i.c. LD_{50} (PFU/LD_{50})	In vivo i.p. LD_{50} (PFU/LD_{50})
Alphaviruses	Semliki Forest virus L10	MBP	6.8×10^7	Not applicable	$10^{6.8}$ (1.1 PFU/LD_{50})
Flaviviruses	West Nile E101	MBP	1.8×10^9	Not being used *in vivo*	
	Yellow Fever 17D	MBP, one passage	1.6×10^7	3×10^4 ($33 PFU/LD_{50}$)	No symptoms with undiluted
	Banzi	MBP	2.0×10^8	Not applicable	$10^{8.4}$ (0.8 PFU/LD_{50})
Bunyaviruses	Caraparu	MLP	Does not plaque	Not applicable	$10^{2.4}$
	Oriboca	MLP	$4x10^6$	Not applicable	$10^{1.2}$ ($2.8x10^3$ PFU/LD_{50})
Arenaviruse	Pichinde CoAn3739	Vero	4.5×10^8	Not being used *in vivo*	
Herpesviruses	HSV-2 MS	RKCC	8.0×10^6	Not applicable	ca. $10^{1.0}$ (ca. 1.0×10^5 PFU/LD_{50})
	HSV-1 Kos	Vero	2.0×10^7	Not being used *in vivo*	

MBP - 10% W/V infant mouse brain pool. MLP - 10% W/V infant mouse liver pool.

Table 3. Dose Response of Ampligen on Peritoneal Macrophage Ectoenzyme Activity

Group	Dose (mg/kg)	Ectoenzyme Specific Activity (n moles/min/mg Protein ± SE) 5'N	APD
Naive	-	10.5 ± 1.4	21.5 ± 0.6
C. parvum	35	0.2 ± 0.1*	2.0 ± 0.1*
Ampligen	8	5.0 ± 2.6	9.9 ± 0.2*
Ampligen	4	3.2 ± 0.9*	9.5 ± 0.3*
Ampligen	2	1.8 ± 0.6*	8.8 ± 0.1*
Ampligen	1	2.7 ± 0.2*	9.2 ± 0.3*

B6C3F1 mice were injected i.p. with C. parvum 7 days before sacrifice. Ampligen was injected i.p. one day before mice were sacrified.
5'N = 5'-nucleotidase; APD = alkaline phosphodiesterase
* = $p < 0.05$ as compared with the naive group.

Table 4. Dose Responses of CL246,738 on peritoneal macrophage antitumor activity and ectoenzyme activity[a]

Group	Dose (mg/kg)	Macrophage Antitumor Activity (% Lysis/Inhibition ± SE)		Ectoenzyme Specific Activity[b] (n moles/min/mg/protein ± SE)	
		LL Target E:T 20:1	B16 Target E:T 20:1	5'N	APD
Naive	-	21.7 ± 4.8	35.3 ± 1.3	32.1 ± 1.3	39.9 ± 0.2
C. parvum	35	100.0 ± 0.0*[c]	100.0 ± 0.0*	0.1 ± 0.1*	2.4 ± 0.04*
CL246,738	800	50.0 ± 5.8*	53.4 ± 1.5*	4.2 ± 0.3*	22.8 ± 0.0*
CL246,738	400	60.4 ± 2.7*	55.8 ± 1.9*	15.9 ± 9.9*	19.3 ± 0.1*
CL246,738	200	55.7 ± 2.1*	52.2 ± 2.4*	5.6 ± 0.2*	23.4 ± 0.03*
CL246,738	100	48.2 ± 2.1*	46.8 ± 2.3*	2.6 ± 1.0*	23.0 ± 0.1*
CL246,738	50	41.6 ± 2.6*	40.7 ± 2.1	2.8 ± 0.4*	28.0 ± 0.2*
CL246,738	25	41.7 ± 1.4*	41.2 ± 0.9	1.2 ± 0.7*	28.2 ± 0.1*

a B6C3F1 mice were injected i.p. with C. parvum 7 days before sacrifice. CL246,738 was administered p.os. one day before sacrifice. Each group represents a pool of 6 mice.

b 5'N = 5' nucleotidase ; APD = alkaline phosphodiesterase

c * = significant at $p < 0.05$ as compared with naive controls.

Table 5. Intrinsic Antiviral Resistance of Res MØ

Virus	Virus yield /cell at Hours after Infection 2-4	24	72-120
Banzi	0.006	0.004	0.001
Semliki Forest	0.13	0.008	0.0005
Pichinde	ND	0.07	0.01
HSV-1	0.11	0.05	0.01
Yellow Fever	0.001	<0.0002	<0.0002
West Nile	0.007	<0.0002	<0.0002
Oriboca	0.01	0.001	ND
Caraparu	<0.0005	ND	<0.005

16 mm diameter wells containing approximately 2 x 10^5 Res MØ were infected with 2-5 m.o.i. for all viruses except Oriboca and Caraparu. Because of the lower titers of those virus stocks, the estimated m.o.i. was 0.04 for Oriboca and <0.001 for Caraparu virus. At the times indicated cell culture fluid and cells (HSV-1 and Pichinde) or fluid only (the other viruses) were harvested for titration of virus, and the cells were counted. During infection there was no apparent decrease in cell numbers and no visible CPE. All viruses except Oriboca and Caraparu were titrated for PFU. The titers of Oriboca and Caraparu represent suckling mouse LD_{50} doses when inoculated by the i.c. route.

Table 6. Effect of Immunomodulator Treatment on Intrinsic Resistance of Macrophages to Infection with HSV-1[a]

Group	Dose	Route	Day	HSV-1 MOI	HSV-1 Mean PFU/cell ± SE at 24hr	HSV-1 Mean CPE ± SE at 24hr	Pichinde Virus MOI	Pichinde Virus Mean PFU/cell ± SE at 48hr
Experimental								
Naive	-	-	-	3.0 ± 0.2	0.016 ± 0.006	1.2 ± 0.5	4.1 ± 1.1	0.06 ± 0.02
C. parvum	35mg/kg	i.p.	-7	3.0	0.023 ± 0.002	3.1 ± 0.2	8.6	0.04 ± 0.02
CL246,738	200mg/kg	p.os	-1	2.6	0.004 ± <0.001	0.5 ± 0	3.2	0.004 ± 0.001
Ampligen	4mg/kg	i.p.	-1	3.2	0.001 ± <0.001	0.2 ± 0	2.4	0.13 ± 0.11
rMuIFN-G	20,000IU	i.p.	-1	2.9	0.003 ± <0.001	0.5 ± 0.3	5.0	0.07 ± 0.02
rHuIFN-A A/D	30,000IU	i.p.	-1	3.2	0.002 ± <0.001	0.2 ± 0	2.4	0.07 ± 0.03
Controls								
Vero Cells (Positive Control)[b]				4.5 ± 0.3	465.3 ± 47.5	3.1 ± 0.3	6.0	44.1 ± 17.0
Thermal Inactivation Control[c]				6.0 ± 0.2	5.5 ± 0.14	-	6.5	3.9 ± 0.7

a Peritoneal macrophages were obtained from B6C3F1 female mice treated with immunomodulators as indicated, the macrophages allowed to adhere for 2hr, non-adherent cells removed by washing and the macrophages infected the next day with HSV-1 (strain Kos) at the indicated multiplicity of infection. The mean PFU were generally determined from 4-10 separate wells.

b Log_{10} PFU/cell of virus replicating in Vero cells, to control for viability of virus in permissive cells.

c Log_{10} PFU/ml of virus incubated in media at 37°C without cells, to control for thermal inactivation of virus.

Table 7. Extrinsic Antiviral Activity of Peritoneal Macrophages Against HSV-1

Treatment of Mice				Mean log_{10} Reduction in HSV-1 Titer[a] ± SE (No. of Exp.)
Drug	Dose	Route	Day	
Naive	-	-	-	0.57 ± 0.23*[b] (7)
C. parvum	35mg/kg	i.p.	-7	1.67 ± 1.22 (3)
TG	2.5mg/kg	i.p.	-5	1.2 ± 0.6* (3)
MVE-2	50mg/kg	i.p.	-1	0.93 ± 0.29* (3)
CL246,738	200mg/kg	p.os	-1	0.70 ± 0.23* (4)
Ampligen	4mg/kg	i.p.	-1	0.67 ± 0.28 (3)
rMuIFN-G	10,000 IU (0.78ug)	i.p.	-1	0.70 ± 0.25 (3)
rHuIFN-A A/D	10,000 IU (0.09ug)	i.p.	-1	1.1 ± 0.22* (4)
rHuINF-A	25,500 IU (0.57ug)	i.p.	-1	1.2 ± 0.20 (2)
rHuIL-1 B	10,000 IU (0.32ug)	i.p.	-1	0.95 ± 0.05* (2)

a The yields of HSV-1 in Vero cells cultured without macrophages ranged from 6.0 - 7.6 log_{10} over the seven experiments

b * = $p < 0.05$ as compared by paired t test with control Vero cells infected with HSV-1 but not cocultured with macrophages. None of the treatment groups were statistically significant from naive or control mice.

Table 8. Extrinsic Antiviral Activity of Peritoneal Macrophages Against Pichinde Virus[a]

MØ Added	Log_{10} PFU ± SE	Log_{10} Difference from Control
None	6.7 ± 0.08	
Res	5.8 ± 0.05*[b]	0.9*[b]
MVE-2	4.8 ± 0.03*	1.9*
CL246,738	5.2 ± 0.15*	1.4*
Ampligen	5.0 ± 0.08*	1.7*

a Vero cells were infected with Pichinde virus, nonadsorbed virus removed with washing, no MØ (control) or MØ from variously treated mice added, and cultures incubated for 48hr. The log_{10} PFU are the yields of total virus in three replicate cultures.

b * = $p < 0.05$ as compared with Vero cells cultured without MØ. The group with immunomodulator activated MØ all also showed significantly enhanced activity as compared with Res MØ.

Table 9. Summary of Immunomodulator Profiles

	Regimen			Splenic NK Cell Activ.	Serum IFN/ Ind.	Peritoneal Macrophage Activation				
	Dose	Route	Schedule			Ectoenzyme Changes 5'N	Ectoenzyme Changes APD	Antitumor Activity	Antiviral Activity[a] Int.	Antiviral Activity[a] Ext.
Placebo (Res)	-	-	-	-	-	-	-	-	+	+
MVE-2	50mg/kg	i.p.	D-1	-	Weak	++	++	++	+	+
C. parvum	35mg/kg	i.p.	D-7	-	Weak	++	++	++	+	+
CL246,738	100mg/kg	p.os	D-1	++	++	+	+	+	+	+
Ampligen	4mg/kg	i.p.	D-1	++	++	+	+	+	+	+
rMuIFN-G	10,000 IU	i.p.	D-1	++	++	+	+	++	+	+
rHuIFN-A A/D	10,000 IU	i.p.	D-1	++	++	+/-	+/-	+/-	+	+

[a] All of the MØ groups have shown intrinsic and extrinsic activity against some virus systems.

Table 10. Summary of Characterization of Total, 2 Hour Adherent, and 24 Hour Adherent Liver Nonparenchymal Cells

	Total Nonparenchymal Cells	Adherent Nonparenchymal Cells	
		2 hours	24 hours
% Macrophages (morphology)	18	ND	68
% LEC (morphology)	40	ND	27
% Lymphocytes (morphology)	30	ND	0
% Parenchymal (morphology)	4	0	0
% Other (morphology)	8	0	0
% Fc IgG receptor + (rosettes)	ND	ND	100
% Phagocytic (IgG-SRBC)	ND	ND	65
% Esterase + (cytochemistry)	100	100	100
% Ovalbumin + (fluorescence)	68	60	0

Table 11. Effect of rMUIFN-G on Peritoneal Macrophage (MØ) and Kupffer cell (KC) Ectoenzyme Activity

Group	Ectoenzyme Specific Activity (in n moles/min/mg protein)	
	5'N	APD
Control PMØ	5.7 ± 0.8	12.6 ± 1.3
rMuIFN-G PMØ	1.8 ± 0.6*	1.4 ± 0.8*
Control KC	8.5 ± 1.8	123.7 ± 7.1
rMuIFN-G KC	3.4 ± 0.2*	95.0 ± 4.4*

B6C3F1 female mice, aged 5-6 weeks old, were injected i.p. 1 day before sacrifice with 10,000 IU rMuIFN-G or with the IFN vehicle. Results are expressed as the mean value ± S.E.M. of triplicate determinations.
*Statistically significant ($p<0.05$) as compared with corresponding control group.

Table 12. Effect of MVE-2 and C. parvum on Kupffer cell (KC) Ectoenzyme Activity

Group	Ectoenzyme Specific Activity (in n moles/min/mg protein)	
	5'N	APD
Naive KC	21.4 ± 1.0	67.5 ± 11.4
MVE-2 KC	8.3 ± 0.3*	180.4 ± 18.0*
C. parvum	2.9 ± 0.1*	66.0 ± 11.5

B6C3F1 female mice, aged 5-6 weeks old, were injected i.p. 6 days before sacrifice with 50 mg/kg MVE-2 or 35 mg/kg C. parvum. Results are expressed as the mean value ± S.E.M.
*Statistically significant ($p < 0.05$) from naive control.

Table 13. Lack of Dose Response of Mab NK1.1 in B6C3F1 Mice

Mab NK1.1	Poly IC	% Cytotoxicity ± SE	(% of Control)
-	-	39.7 ± 4.8	
50 ul	-	11.4 ± 0.8	(29%)
100 ul	-	9.0 ± 0.5	(23%)
200 ul	-	8.7 ± 0.3	(22%)
-	+	68.3 ± 5.4	
50 ul	+	42.6 ± 4.3	(62%)
100 ul	+	40.8 ± 4.8	(60%)
200 ul	+	46.3 ± 5.1	(68%)

Three B6C3F1 female mice were inoculated i.p. with the the indicated amounts of Mab NK1.1 or were untreated. Two days later, mice were injected i.p. with 100 ug (5mg/kg) of poly IC or physiological saline. The next day spleens were removed and cytotoxic activity against Yac-1 cells at an effector: target ratio of 200:1 was measured. The % cytotoxicity is the arithmetic mean ± SE.

Table 14. Effect of combined treatment with Mab NK1.1 and Poly IC on NK cell activity

Poly IC	Mab	Poly IC	% Cytotoxicity ± SE	% Control
-	-	-	20.0 ± 1.5	
-	+	-	1.1 ± 0.3	5.5%
+	-	-	37.3 ± 3.2	
+	+	-	2.3 ± 0.4	6.1%
-	-	+	54.3 ± 1.2	
-	+	+	25.5 ± 2.4	47.0%
+	-	+	47.0 ± 3.0	
+	+	+	35.7 ± 3.5	76.0%

Three B6C3F1 female mice were injected i.p. with 100 ug of Poly IC on day -4 or were untreated. One day later (day -3) mice were inoculated i.p. with 100 ul of Mab NK1.1 or physiological saline. Two days later mice were injected i.p. with 100 ug Poly IC or were untreated. On day 0 spleens were removed and cytotoxic activity against Yac-1 cells at an effector: target ratio of 200:1 was measured

Table 15. Effect of repeated NK 1.1 treatment regimens on spontaneous and PolyI:C induced splenic NK activity

Group	% Cytotoxicity Days of Treatment -10	-20,-10	-24,-14,-4
Control	22.9	60.3	66.3
Mab NK 1.1	12.5 (54%)	12.7 (20)%	17.6 (26%)
Poly I:C	44.7	71.5	56.3
Mab NK 1.1 + PolyI:C	23.1 (52%)	35.1 (49%)	31.4 (56%)

Each group contained 3 B6C3F1 female mice. 100ul of Mab NK 1.1 was inoculated i.p. according to indicated schedule. Mice were sacrified at day 0, their spleens were removed and NK activity against YAC-1 target cells was measured at an effector to target ratio of 200:1. PolyI:C was injected i.p. one day before sacrifice.

Table 16. Kinetics of Effects of i.v. Administration of Liposomes Containing DMDP on Blood and Peritoneal Cell Populations[a]

Treatment Group	Days After	WBC x 10^5 ± SE/ml Total	MØ	LY	PMN	PC x 10^6 ± SE/mouse Total	MØ	SCx10^7 ± SE/mouse Total	MØ
Saline	1	113.6 ± 33.6	15.3 ± 3.9	80.2 ± 27.8	18.0 ± 6.7	1.7 ± 0.4	1.4 ± 0.3	4.5 ± 0.3	0.23 ± 0.04
Free L	1	154.7 ± 35.8	22.4 ± 6.8	123.2 ± 37.0	30.0 ± 9.8	2.4 ± 0.2	2.0 ± 0.1	3.7 ± 0.4	0.17 ± 0.03
DMDP L	1	420.2 ± 56.4*[b]	24.2 ± 4.1	308.0 ± 35.8*	72.6 ± 15.6*	2.5 ± 0.2	1.8 ± 0.2	1.0 ± 0.01	0.06 ± 0.01*
Saline	4	83.8 ± 13.0	4.2 ± 2.1	51.0 ± 4.9	17.3 ± 2.6	4.0 ± 0.4	3.3 ± 0.3	9.4 ± 0.9	0.26 ± 0.03
Free L	4	123.7 ± 6.5	6.3 ± 1.7	85.4 ± 5.8	32.3 ± 5.2	4.6 ± 0.3	3.8 ± 0.3	11.7 ± 0.6	0.30 ± 0.10
DMDP L	4	199.0 ± 44.9*	8.1 ± 2.3	138.3 ± 24.8*	78.3 ± 20.6*	4.9 ± 0.7	3.8 ± 0.7	9.2 ± 1.4	0.43 ± 0.10
Saline	6	80.9 ± 22.8	2.4 ± 0.7	57.9 ± 19.3	20.8 ± 4.0	3.4 ± 0.1	2.6 ± 0.1	5.6 ± 0.8	0.25 ± 0.08
Free L	6	78.3 ± 18.5	2.9 ± 0.8	48.9 ± 10.8	26.3 ± 8.0	3.2 ± 0.2	2.4 ± 0.1	7.9 ± 1.4	0.51 ± 0.24
DMDP L	6	230.0 ± 14.0*	6.0 ± 1.6	144.2 ± 10.2*	78.0 ± 9.4*	2.9 ± 0.3	2.2 ± 0.1	1.2 ± 0.1	0.04 ± 0.01

a CD1 adult female mice were injected twice i.v. with saline, free liposomes or liposomes encapsulating DMDP on days -1 and -3 relative to the first day (day 1) on which cell populations were analyzed. Each group generally consisted of data from 6 individual mice. WBC = white blood cells, PC = peritoneal cells, and SC = spleen cells.

b * = $p < 0.05$ as compared with saline controls

Table 17. Lack of Effect of i.v. DMDP Liposomes on Peritoneal Cells

Exp. Group	Exp. #	PEC/Mouse ($x10^6$) MØ	PEC/Mouse ($x10^6$) LY	Ectoenzyme SA 5'N	Ectoenzyme SA APD
Naive	1	3.9 ± 0.2	1.5 ± 0.03	10.2 ± 3.0	19.4 ± 1.1
	2	1.8 ± 0.1	3.1 ± 0.5	11.7 ± 7.0	16.0 ± 1.8
NaCl	1	3.5 ± 0.5	1.6 ± 0.3	15.2 ± 5.3	22.7 ± 2.0
	2	2.0 ± 0.3	3.1 ± 0.4	10.1 ± 1.3	20.2 ± 1.9
Free Lip -3, -1	1	4.1 ± 0.3	1.3 ± 0.2	14.0 ± 2.7	18.6 ± 0.5
	2	ND	ND	ND	ND
DMDP Lip -3, -1	1	3.9 ± 0.6	1.6 ± 0.3	12.7 ± 1.9	19.1 ± 1.7
	2	2.0 ± 0.5	2.6 ± 0.2	8.6 ± 1.4	19.4 ± 2.5
DMDP Lip -2	1	ND	ND	ND	ND
	2	1.9 ± 0.3	2.8 ± 0.2	8.2 ± 1.4	20.4 ± 0.8

There were no significant differences in any groups as compared with naive or NaCl control groups.

Table 18. Effect of DMDP Liposomes on WBC, PEC and NK cells

Exp. Group	Exp. #	WBC/ml (#x10^5) ± SE			NK cell Cytotoxicity (% ± S.E. at 200:1)	
		Mono	Ly	PMN	Spontaneous	+ IFN
Naive	1	2.0 ± 0.7	60.0 ± 6.7	17.1 ± 3.5	15.0 ± 3.6	40.1 ± 5.7
	2	ND	ND	ND	22.1 ± 4.2	50.5 ± 4.3
NaCl	1	6.0 ± 1.2	58.0 ± 10.1	17.0 ± 2.3	21.6 ± 1.9	51.0 ± 3.9
	2	2.5 ± 0.6	43.2 ± 4.4	8.6 ± 1.1	31.5 ± 1.0	53.2 ± 2.1
Free Lip. -3, -1	1	3.8 ± 1.0	54.0 ± 7.7	32.3 ± 8.8	19.5 ± 2.2	46.6 ± 3.0
	2	ND	ND	ND	ND	ND
DMDP Lip. -3, -1	1	5.2 ± 0.9	136.0 ± 13.7*	49.7 ± 9.6*	3.0 ± 0.5*	10.6 ± 2.9*
	2	6.2 ± 1.4*	107.6 ± 15.3*	32.0 ± 13.6*	6.2 ± 1.9*	17.2 ± 3.8*
DMDP Lip. -2	1	ND	ND	ND	ND	ND
	2	1.5 ± 0.5	62.4 ± 8.2	19.2 ± 8.3	17.3 ± 2.6	26.5 ± 4.6*

* $p < 0.05$ by ANOVA and Tukey post hoc test as compared with naive group for all data in Exp. 1 and 2, except for comparison with the NaCl group for WBC data for Exp. 2.

Table 19. Effect of i.v. DMDP Liposomes on Resistance to HSV-2 Infection in CD-1 Mice

Virus Dilutions		Saline	Free Lip. Day -3, -1	DMDP Lip. Day-2	DMDP Lip. Day-3, -1
			Exp. 1 HSV-2 i.p. infection		
-1.0	% Dead	80.0	100.0	100.0	100.0
	MST	11.0	7.8	7.8	8.0
-1.5	% Dead	80.0	100.0	100.0	100.0
	MST	11.2	7.8	9.2	8.0
-2.0	% Dead	80.0	80.0	100.0	100.0
	MST	12.8	11.8	7.0	9.0
-2.5	% Dead	100.0	100.0	100.0	100.0
	MST	10.0	11.8	9.8	9.2
-3.0	% Dead	40.0	20.0	60.0	80.0
	MST	18.4	21.2	19.8	13.0
-3.5	% Dead	40.0	60.0	60.0	75.0
	MST	19.8	16.6	16.6	13.5
			Exp. 2 HSV-2 i.v. infection		
-1.0	% Dead	100.0	ND	100.0	ND
	MST	7.5	ND	7.8	ND
-2.0	% Dead	100.0	ND	17.0	ND
	MST	10.7	ND	17.8	ND
-3.0	% Dead	17.0	ND	17.0	ND
	MST	18.0	ND	18.5	ND
-4.0	% Dead	0	ND	17.0	ND
	MST	>20.0	ND	18.5	ND
			Exp. 3 HSV-2 i.v. infection		
-1.0	% Dead	100.0	100.0	ND	100.0
	MST	5.5	6.0	ND	4.0
-2.0	% Dead	100.0	100.0	ND	100.0
	MST	6.0	6.5	ND	5.0
-3.0	% Dead	100.0	100.0	ND	100.0
	MST	7.0	7.0	ND	6.5
-4.0	% Dead	33.0	100.0	ND	100.0
	MST	>21.0	10.0	ND	9.0
-5.0	% Dead	17.5	33.3	ND	83.3
	MST	>21.0	>21.0	ND	9.0

CD-1 mice were treated as indicated, infected i.p. or i.v. with HSV-2, mortality recorded and median survival calculated. Each group has 5-6 mice.

Table 20. Effect of DMDP Liposomes on Splenic NK Cell Activity[a]

Treatment Group	Days after	Splenic NK Cell Cytotoxicity ± SE (% of control) Spontaneous		+ Interferon	
Saline	1	15.8 ± 2.8		27.3 ± 2.9	
Free Lip.		17.2 ± 2.0	(108%)	23.7 ± 1.4	(87%)
DMDP Lip.		9.4 ± 3.3	(59%)*[b]	16.9 ± 6.2*	(62%)*
Saline	4	37.3 ± 1.2		54.7 ± 3.7	
Free Lip.		39.3 ± 3.0	(105%)	47.7 ± 1.9	(87%)
DMDP Lip.		42.3 ± 1.4	(114%)	43.7 ± 3.2	(80%)

a Mice were treated as shown for Table 16

b * = $p < 0.05$ for NK cell activity expressed as percent of controls as compared with the combined controls.

Table 21. Effect of i.v. Administration of DMDP Liposomes on Resistance to i.v. infection with Listeria monocytogenes (strain EGD) in CD-mice[a]

Group	Listeria CFU	% Dead	Median Survival Time
Saline	2.3×10^6	100.0	3.0
Free Liposomes	2.3×10^6	100.0	3.5
DMDP Lipsomes	2.3×10^6	100.0	2.5
Saline	7.8×10^5	100.0	3.6
Free Lipsomes	7.8×10^5	100.0	3.6
DMDP Liposomes	7.8×10^5	100.0	2.5*[b]
Saline	2.3×10^5	83.3	6.0
Free Liposomes	2.3×10^5	100.0	4.5
DMDP Lipsomes	2.3×10^5	100.0	2.5*
Saline	7.8×10^4	16.7	>14.0
Free Liposomes	7.8×10^4	16.7	>14.0
DMDP Liposomes	7.8×10^4	100.0*	2.5*
Saline	2.3×10^4	33.3	>14.0
Free Liposomes	2.3×10^4	33.3	>14.0
DMDP Liposomes	2.3×10^4	100.0*	2.5*

a CD-1 female mice, aged 6 weeks, were treated i.v. as indicated on days -3 and -1 before i.v. infection with the indicated dose of Listeria. Each group contained 6 mice.

b * = Statistically signficiant ($p < 0.05$) as compared with saline and free liposome control groups.

Table 22. Effect of i.p. Administration of Ricin-Liposomes on Resistance of Mice to i.p. Infection with *Listeria monocytogenes*[a]

Treatment		Log10 CFU Listeria in		
89Sr	R-L	Spleen	Liver	Peritoneum
-	-	1.6 ± 0.4	<1.0 ± 0	<0.7 ± 0
-	+	3.2 ± 1.5 (1.6)[b]	2.9 ± 1.0 (>2.9)	2.7 ± 1.0 (>2.0)
+	-	1.5 ± 0.4	1.7 ± 0.7	0.9 ± 0.1
+	+	4.4 ± 0.3 (2.9)	4.3 ± 0.5 (2.6)	3.3 ± 0.1 (2.4)

a CD1 female mice were injected i.v. with ^{88}Sr or ^{89}Sr, and i.p. with free liposomes or ricin liposomes (R-L) 13 days later. The next day mice were challenged with approximately an LD_{50} dose of Listeria, and were sacrified 28 hours later. Spleens and livers were obtained, weighed, frozen at -70°C, homogenized and the titer of Listeria per organ determined by colony formation (CFU) on blood agar. The peritoneal compartment was lavaged with 5ml of PBS, frozen and the titer of Listeria per 0.5 ml determined. Each group consisted of 3-5 mice.

b Numbers in parenthesis = $\log_{10}$ difference in geometric means of the R-L groups compared with the respective control free lipsomes groups.

Table 23. Effect of Immunomodulators on Banzi Flavivirus Infection[a]

Drug	Drug Treatment: Dose	Schedule (days)	Route	Vehicle	Mortality: Dead/Total	Mortality: (%)	Survival: MST	Survival: VR[b]
Positive Control Drugs								
MVE-2	50 mg/kg	-1	i.p.	PBS	0/10	(0%)*[c]	>16.0*	>1.8*
C. parvum	35 mg/kg	-7	i.p.	PBS	3/10	(30%)*	17.9*	2.2*
CL246738: Prophylactic and/or Therapeutic Treatment								
CL246,738	6 mg/kg	-1	p.os	H_2O	2/10	(20%)*	>16.0*	>1.8*
CL246,738	50 mg/kg	0 to +6	p.os	H_2O	2/10	(20%)	>20.0*	>2.0*
CL246,738	200 mg/kg	+1	p.os	H_2O	8/10	(80%)	10.8*	1.2*
CL246,738	200 mg kg	+3	p.os	H_2O	10/10	(100%)	8.3	1.0
CL246,738	200 mg/kg	+1,2,4,6	p.os	H_2O	9/10	(90%)	9.8	1.1
Ampligen: Prophylactic and/or Therapeutic Treatment								
Ampligen	4 mg/kg	-1	i.p.	NaCl	3/10	(30%)*	>19.0*	>2.2*
Ampligen	4 mg/kg	0 to +2	i.p.	NaCl	2/10	(20%)*	>19.0*	>2.2*
Ampligen	4 mg/kg	0 to +6	i.p.	NaCl	0/10	(0%)*	>16.0*	>1.8*
Ampligen	4 mg/kg	+1 to +4	i.p.	NaCl	9/10	(90%)	11.7*	1.2*
Ampligen	4 mg/kg	+1 to +6	i.p.	NaCl	2/10	(20%)*	>16.0*	>1.8*
Ampligen	4 mg/kg	+1 to +6	i.p.	NaCl	6/10	(60%)	13.5*	1.4*
Ampligen	4 mg/kg	+2 to +6	i.p.	NaCl	10/10	(100%)	11.0*	1.2*
Ampligen	4 mg/kg	+2 to +8	i.p.	NaCl	5/10	(50%)	>20.0*	>2.0*
Recombinant Derived Biologicals: Prophylactic and/or Therapeutic Treatment								
rHuIFN-A A/D	67,000 IU(0.35 ug)	0	i.p.	PBS/0.2% BSA	6/10	(60%)	10.8*	1.4*
rHuIFN-A A/D	10,000 IU(0.15 ug)	0	i.p.	PBS/0.2% BSA	2/10	(20%)*	>20.0*	>2.1*
rHuIFN-A A/D	10,000 IU(0.15 ug)	0 to +6	i.p.	PBS/0.2% BSA	0/10	(0%)*	>16.0*	>1.8*
rHuIFN-A A/D	67,000 IU(0.35 ug)	0,+1,+2,+4,+6	i.p.	PBS/0.2% BSA	1/11	(9%)*	>19.0*	>2.4*
rHuIFN-A A/D	10,000 IU(0.15 ug)	0 to +2	i.p.	PBS/0.2% BSA	0/10	(0%)*	>20.0*	>2.1*
rHuIFN-A A/D	10,000 IU(0.15 ug)	+1 to +6	i.p.	PBS/0.2% BSA	7/10	(70%)	13.3*	1.5*
rHuIFN-A A/D	10,000 IU(0.16 ug)	+1 to +6	i.p.	PBS/0.2% BSA	4/10	(40%)*	>20.0*	>2.1*
rHuIFN-A A/D	67,000 IU(0.35 ug)	+1	i.p.	PBS/0.2% BSA	10/10	(100%)	9.8*	1.2*

(Continued on next page)

Table 23. Effect of Immunomodulators on Banzi Flavivirus Infection[a] (Continued)

Drug Treatment					Mortality		Survival	
Drug	Dose	Schedule (days)	Route	Vehicle	Dead/ Total	(%)	MST	VR[b]
rHuTNF-A	10,000 IU(0.57 ug)	-1 to +6	i.p.	PBS/0.2% BSA	8/10	(80%)	10.8	1.1
rHuCSF-M	50ug	-1 to +6	i.p.	PBS/0.2% BSA	10/10	(100%)	9.6	1.0
Pyrimidinones: Prophylaxis								
ABPP	200	-1	i.p.	1% CMC	5/20	(25%)*	>19.0*	>2.2*
ABPP	200	-1	p.os	1% CMC	2/4	(50%)*	>19.0*	>2.2*
AIPP	200	-1	i.p.	1% CMC	3/11	(27%)*	>19.0*	>2.2*
AIPP	200	-1	p.os	1% CMC	9/10	(90%)	9.7*	1.1*
ABMP	200	-1	i.p.	1% CMC	6/10	(60%)*	10.0*	1.2*
ABMP	200	-1	p.os	1% CMC	5/10	(50%)*	>19.0*	>2.2*

a B6C3F1 female mice, aged 5-6 weeks old, were treated as indicated and infected on day 0 with 2.4-7.0 PFU (5-20 LD_{50} doses) of Banzi virus. The percent mortality in control groups ranged from 90-100%, and the MST from 8-9 days.

b * = MST of experimental group/MST of control group

c = * Statistically significant ($p<0.05$) as compared with the Placebo group.

Table 24. Effect of Immunomodulators on Semliki Forest Virus Infection[a]

Drug Treatment					Mortality		Survival	
Drug	Dose	Schedule (days)	Route	Vehicle	Dead/ Total	(%)	MST	VR[b]
Positive Control Drugs								
C. parvum	35 mg/kg	-7	i.p.	NaCl	0/10	(0%)*[c]	>14.0*	>3.5*
MVE-2	50 mg/kg	-1	i.p.	PBS	0/10	(0%)*	>14.0*	>3.5*
Synthetics Immunomodulators								
Ampligen	4 mg/kg	-1	i.p.	NaCl	0/10	(0%)*	>14.0*	>3.5*
Ampligen	4 mg/kg	0 to +6	i.p.	NaCl	0/10	(0%)*	>14.0*	>3.5*
CL246,738	30 mg/kg	-1	p.os	H_2O	0/10	(0%)*	>14.0*	>3.5*
ABPP	200 mg/kg	-1	p.os	1% CMC	0/10	(0%)*	>14.0*	>3.5*
AIPP	200 mg/kg	-1	i.p.	1% CMC	0/10	(0%)*	>14.0*	>3.5*
ABMP	200 mg/kg	-1	i.p.	1% CMC	2/10	(20%)*	>14.0*	>3.5*
Recombinant Derived Biologicals								
rHuIFN-A A/D	2,700IU (0.13 ug)	-1, +2 hr	i.p.	PBS/0.2% BSA	2/10	(20%)*	>14.0*	>3.0*
rHuIFN-A A/D	2,700IU (0.13 ug)	0	i.p.	PBS/0.2% BSA	2/10	(20%)*	>14.0*	>3.0*
rHuIFN-A/D	10,000IU (0.16 ug)	+1 to +6	i.p.	PBS/0.2% BSA	3/10	(30%)*	>20.0*	>3.3*
rMuIFN-G	2,400IU (0.70 ug)	-1, +2 hr	i.p.	PBS/0.2% BSA	3/10	(30%)*	>14.0*	>3.0*
rMuIFN-G	2,400IU (0.70 ug)	0	i.p.	PBS/0.2% BSA	8/10	(80%)	6.3*	1.3*
rMuIFN-G	10,000IU (0.96 ug)	0 to +6	i.p.	PBS/0.2% BSA	2/10	(20%)*	>20.0*	>3.3*
rMuIFN-G	10,000IU (0.96 ug)	+1 to +6	i.p.	PBS/0.2% BSA	9/10	(90%)	6.5	1.1
rHuCSF-M	50 ug	-1 to +6	i.p.	PBS/0.2% BSA	7/9	(78%)	8.0	1.3*
Microbial Derived Biologicals								
MPL+TDM	100ug+100ug	-3,0,+3	i.p.	2% squalene	2/10	(20%)*	>20.0*	>3.3*
MPL+TDM+CWS	100ug+100ug+100ug	-3,0,+3	i.p.	2% squalene	2/10	(20%)*a	>20.0*	>3.3*

a B6C3F1 female mice, aged 4-6 weeks old, were treated as indicated and infected on day 0 i.p. with 3.5-7.6 PFU (8.5-10 LD_{50} doses) of Semliki Forest virus. Mortality ranged from 80-100% and the MST from 4.0 to 6.3 days.

b VR = MST of experimental group/MST of control group

c * Statistically significant ($p < 0.05$) as compared with the corresponding Placebo control groups.

Table 25. Effect of Immunomodulators on Caraparu Bunyavirus Infection[a]

Drug Treatment					Mortality		Survival	
Drug	Dose	Schedule (days)	Route	Vehicle	Dead/ Total	(%)	MST	VR[b]
Positive Control Drug								
MVE-2	50 mg/kg	-1	i.p.	PBS	3/10	(30%)*	[c]>16.0*	>3.0*
Synthetic Immunomodulators								
Ampligen	4 mg/kg	-1 to +6	i.p.	NaCl	0/10	(0%)*	>15.0*	>2.6*
Ampligen	4 mg/kg	-1 to +6	i.p.	NaCl	7/10	(70%)	6.6*	1.2*
Ampligen	4 mg/kg	-1 to +6	i.p.	NaCl	10/10	(100%)	5.7	1.1
Ampligen	4 mg/kg	0 to +6	i.p.	NaCl	9/10	(90%)	6.7*	1.3*
Ampligen	4 mg/kg	0 to +6	i.p.	NaCl	7/10	(70%)	6.3	1.2
Ampligen	8 mg/kg	-1,0	i.p.	NaCl	10/10	(100%)	5.6	1.0
ABPP	200 mg/kg	-1	i.p.	1% CMC	9/10	(90%)	6.2	1.2
AIPP	200 mg/kg	-1	i.p.	1% CMC	9/10	(90%)	5.0	0.9
ABMP	200 mg/kg	-1	i.p.	1% CMC	10/10	(100%)	5.8	1.1
CL246,738	400 mg/kg	-1	p.os	H_2O	1/10	(10%)	>15.0*	>2.6*
CL246,738	400 mg/kg	-1	p.os	H_2O	7/10	(70%)	7.5*	1.4*
CL246,738	400 mg/kg	-1	p.os	H_2O	8/9	(89%)	6.6*	1.2*
CL246,738	400 mg/kg	0 to +6	p.os	H_2O	10/10	(100%)[d]	6.3*	1.2*
Recombinant Derived Biologicals								
rHuIFN-A A/D	12,000 IU (0.47ug)	-1 to +6	i.p.	PBS/0.2% BSA	3/10	(30%)*	>15.0*	>2.6*
rHuIFN-A A/D	20,000 IU (0.30ug)	-1 to +6	i.p.	PBS/0.2% BSA	8/10	(80%)	5.7	1.1
rHuIFN-A A/D	12,000 IU (0.47ug)	0 to +6	i.p.	PBS/0.2% BSA	7/10	(70%)	7.3*	1.3*
rHuIFN-A A/D	20,000 IU (0.30ug)	0 to +6	i.p.	PBS/0.2% BSA	10/10	(100%)	5.6	1.1

(continued on next page)

Table 25. Effect of Immunomodulators on Caraparu Bunyavirus Infection[a] (continued)

Drug Treatment					Mortality		Survival	
Drug	Dose	Schedule (days)	Route	Vehicle	Dead/ Total	(%)	MST	VR[b]
rMuIFN-G	19,500 IU (0.88ug)	-1 to +6	i.p.	PBS/0.2% BSA	5/10	(50%)	>15.0*	>2.6*
rMuIFN-G	40,000 IU (3.11ug)	-1 to +6	i.p.	PBS/0.2% BSA	5/10	(50%)	>20.0*	>3.8*
rMuIFN-G	19,500 IU (0.88ug)	0 to +6	i.p.	PBS/0.2% BSA	5/10	(50%)	>15.0*	>2.6*
rMuIFN-G	40,000 IU (3.11ug)	0 to +6	i.p.	PBS/0.2% BSA	5/10	(50%)	>20.0*	>3.8*
rHuIL-1-B	10,000 IU (0.33ug)	-1 to +6	i.p.	PBS/0.2% BSA	10/10	(100%)	5.0	0.9
rHuTNF-A	10,000 IU (0.57ug)	-1 to +6	i.p.	PBS/0.2% BSA	7/10	(70%)	5.8*	1.1*

a B6C3F1 female mice, aged 5 weeks old, were treated as indicated and infected on day 0 with 10-20 LD_{50} doses of Caraparu virus. The percent mortality in control groups ranged from 63-100% and the MST from 5.3 to 5.7 days.

b VR = MST of experimental group/MST of control group

c * Statistically significant ($p<0.05$) as compared with the corresponding placebo control group.

d There was drug toxicity at this dose and schedule, 40% mortality in mice treated with drug.

Table 26. Effect of Immunomodulators on HSV-2 (Strain MS) Infection[a]

Drug	Drug Treatment: Dose	Schedule (days)	Route	Vehicle	Mortality: Dead/ Total	Mortality: (%)	Survival: MST	Survival: VR[b]
Positive Control Drugs								
MVE-2	50 mg/kg	-1	i.p.	PBS	0/8	(0%)*[c]	>21.0*	>2.0*
C. parvum	35 mg/kg	-7	i.p.	NaCl	0/10	(0%)*	>21.0*	>2.1*
Synthetic Immunomodulators								
Ampligen	4 mg/kg	-1 to +6	i.p.	NaCl	1/10	(10%)*	>21.0*	>2.1*
Ampligen	4 mg/kg	0 to +6	i.p.	NaCl	1/10	(10%)*	>21.0*	>2.1*
ABPP	200 mg/kg	-1	i.p.	1 % CMC	1/10	(10%)*	>21.0*	>2.1*
ABMP	200 mg/kg	-1	i.p.	1 % CMC	6/10	(60%)	10.5	1.0
AIPP	200 mg/kg	-1	i.p.	1 % CMC	2/10	(20%)*	>21.0*	>2.1*
HDPP	3 mg/kg	-2 to 0	p.os	H_2O	8/10	(80%)	10.0	1.0
Recombinant Derived Biologicals								
rHuIFN-A A/D	10,000IU (0.93 ug)	0 to +6	i.p.	PBS/0.2% BSA	2/10	(20%)*	>21.0*	>2.1*
rHuIFN-A A/D	6,700IU (0.03 ug)	-1, -4hr	i.p.	PBS/0.2% BSA	3/10	(30%)*	>21.0*	>2.6*
rHuIFN-A A/D	10,000IU (0.93 ug)	+1 to +2	i.p.	PBS/0.2% BSA	1/10	(10%)*	>21.0*	>2.1*
rMuIFN-G	20,000IU (0.76 ug)	-1	i.p.	PBS/0.2% BSA	6/10	(60%)	13.1*	1.6*
rMuIFN-G	11,000IU (0.71 ug)	-1, -4hr	i.p.	PBS/0.2% BSA	3/10	(30%)*	>21.0*	>2.6*
rMuIFN-G	10,000IU (0.83 ug)	-1 to +2	i.p.	PBS/0.2% BSA	2/10	(20%)*	>21.0*	>2.1*
rMuIFN-G	20,000IU (0.76 ug)	0	i.p.	PBS/0.2% BSA	9/10	(90%)	8.7	1.0
rMuIFN-G	10,000IU (0.83 ug)	0 to +6	i.p.	PBS/0.2% BSA	1/10	(10%)*	>21.0*	>2.1*
rHuIL-1-B	10,000IU (0.33 ug)	-1 to +6	i.p.	PBS/0.2% BSA	3/10	(30%)*	>21.0*	>2.6*
rHuIL-1-B	10,000IU (0.33 ug)	-1 to +6	i.p.	PBS/0.2% BSA	7/10	(70%)	10.5	1.1
rHuTNF-A	25,000IU (0.57 ug)	-1 to +6	i.p.	PBS/0.2% BSA	9/10	(90%)	9.3	0.9
rHuTNF-A	25,000IU (0.57 ug)	-1, -4hr	i.p.	PBS/0.2% BSA	8/10	(80%)	13.0	1.3
rHuCSF-M	50 ug	-1 to 0	i.p.	PBS/0.2% BSA	8/10	(80%)	13.0	1.3

a B6C3F1 female mice aged 4-7 weeks old, were treated as indicated and infected on day 0 i.p. with 4-10 LD_{50} doses. The percent mortality of controls ranged from 62-90%, and the MST from 8.2 to 10.0 days.

b VR = MST of experimental group/MST of control group

c *Statistically significant ($p < 0.05$) as compared with the corresponding Placebo control groups.

Table 27. Effect of ribavirin on alpha-, flavi-, and bunya-virus infections[a]

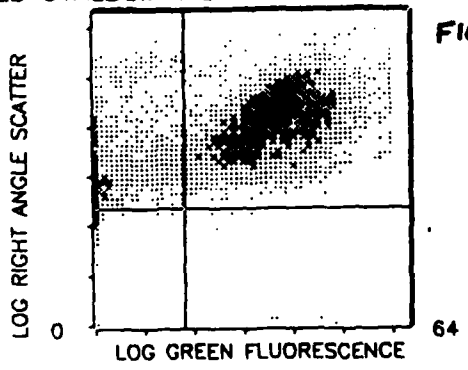
Ribavirin Treatment 100mg/kg)			SFV			Banzi			Caraparu		
Schedule (days)	Route	Vehicle	Dead/ Total(%)	MST	VR	Dead/ Total(%)	MST	VR	Dead/ Total(%)	MST	VR
Placebo	i.p.	PBS	13/14 (93%)	6.3		15/15 (100%)	9.8		14/15		5.4
-1 to +6,+8,+10 +12,+14,+16	i.p.	PBS	8/10 (80%)	6.6	1.0	6/10 (60%)*[b]	15.0*	1.5*	0/10 (0%)*	>20.0*	>3.7*
0 to +6,+8,+10 +12,+14,+16	i.p.	PBS	9/10 (90%)	6.6	1.0	6/10 (60%)*	10.8*	1.1*	0/10 (0%)*	>20.0*	>3.7*

a B6C3F1 female mice, aged 5 weeks old, were treated as indicated and infected on day 0 i.p. with SFV (2.5 LD_{50} doses), Banzi (5 LD_{50} doses) or Caraparu virus (4 LD_{50} doses).

b * Statistically significant ($p < 0.05$) as compared with the Placebo control group.

FIG 1. FITC CONJUGATED OVALBUMIN STAINING OF LIVER NON-PARENCHYMAL CELLS

FIG. 1a



2 PARAMETER ANALYSIS OF TOTAL NON-PARENCHYMAL CELL PREPARATION
PERCENT POSITIVE FOR OVALBUMIN STAINING 68.68%

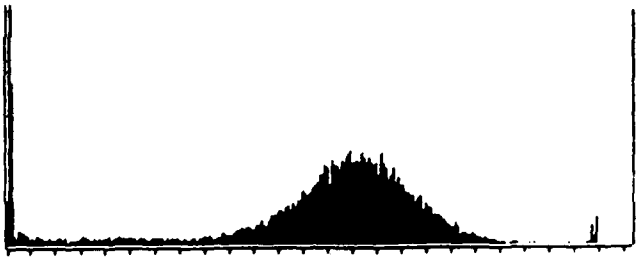
FIG. 1b

KC- 2 HR NON ADHERENT FRACTION
PERCENT POSITIVE FOR OVALBUMIN STAINING = 77.72

FIG. 1c

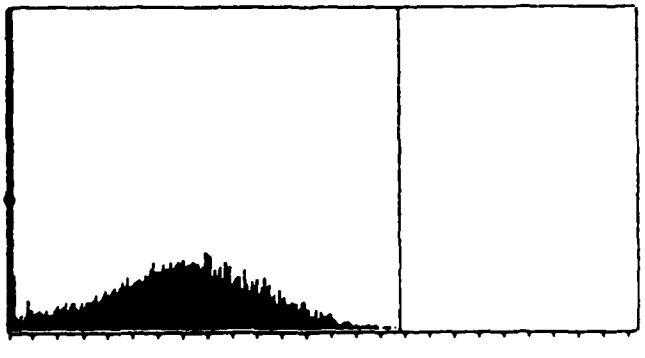
KC- 24 HR NON ADHERENT FRACTION
PERCENT POSITIVE FOR OVALBUMIN STAINING = 88.56

FIG. 1d



SUMMATION OF 2HR & 24 HR NON-ADHERENT FRACTION
MEAN LOG FLUORESCENCE INTENSITY = 148.0

FIG. 1e



24 HOUR ADHERENT POPULATION (STAINED & TRYPSINIZED)
MEAN LOG FLUORESCENCE INTENSITY = 61.15

FIG 2. Fc RECEPTOR ANALYSIS OF NON-PARENCHYMAL CELLS

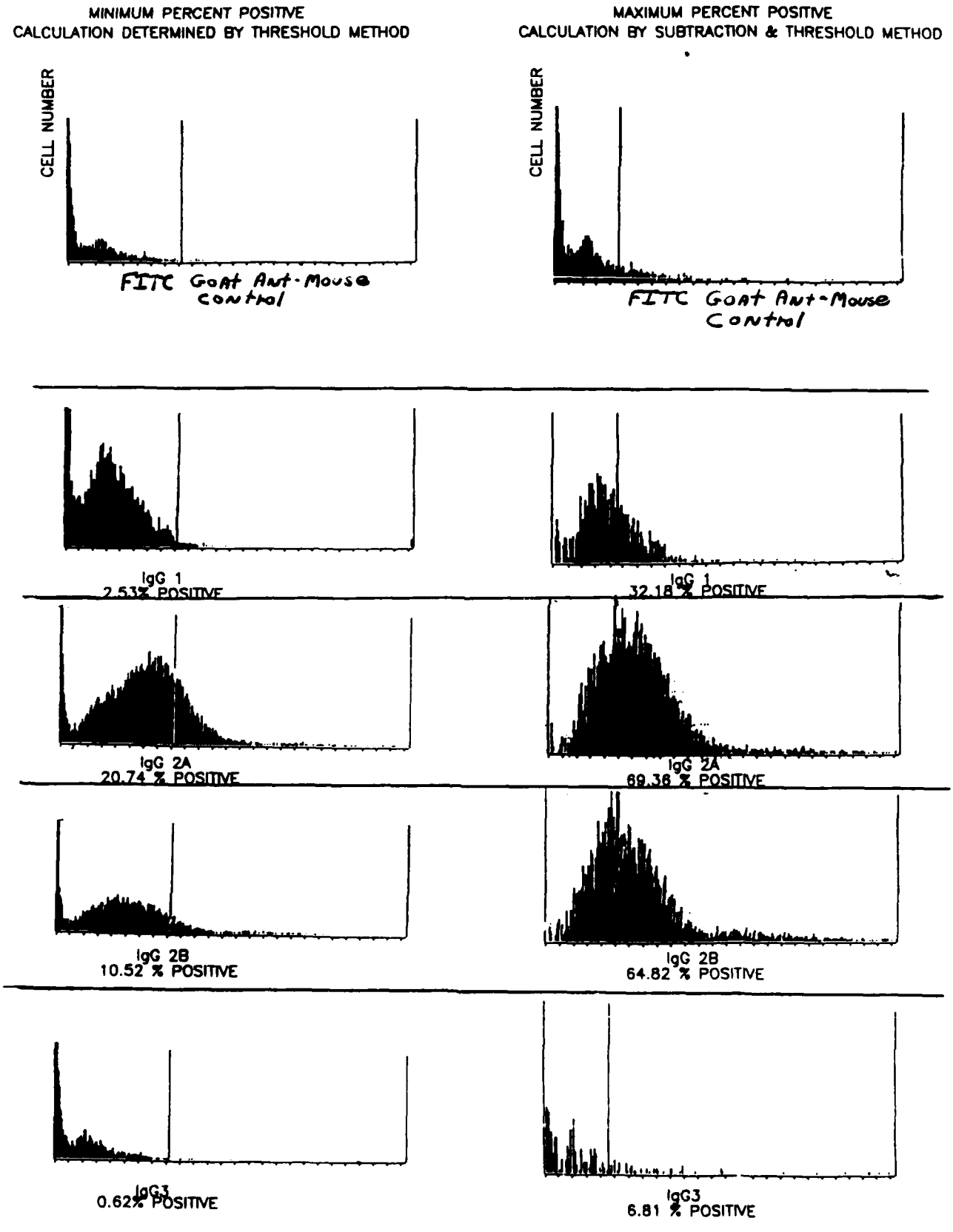
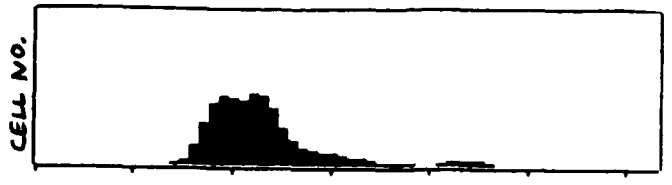


FIG3. PERITONEAL CELLS

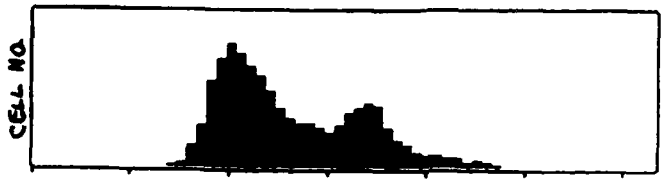
LOG RIGHT ANGLE LIGHT SCATTTER 1 PARAMETER ANALYSIS

PEC– 2 HR NON ADHERENT FRACTION

PEC– 24 HR NON ADHERENT FRACTION

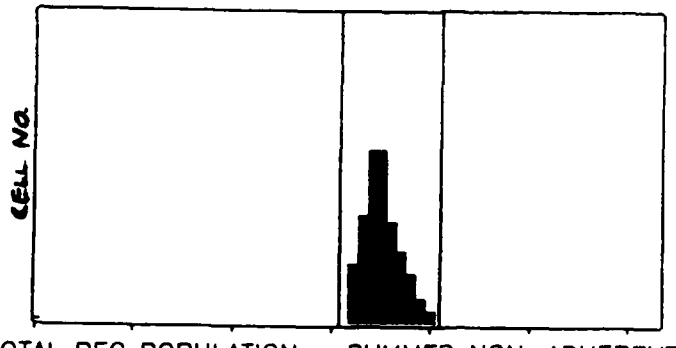


SUMMATION OF 2HR & 24 HR NON–ADHERENT FRACTION



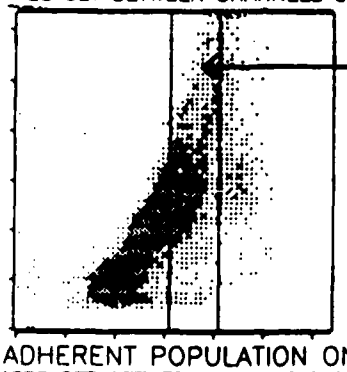
Total input peritoneal population

PEC– TOTAL NON–ADHERED POPULATION (TIME 0)



24 hour Adherent Peritoneal Cells

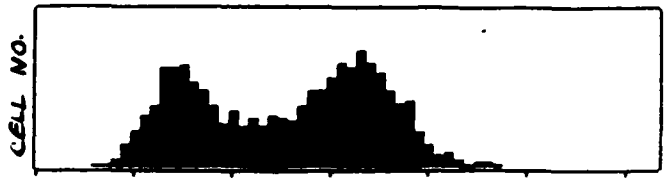
TOTAL PEC POPULATION – SUMMED NON–ADHERENT FRACTIONS
GATES SET BETWEEN CHANNELS 31 TO 41



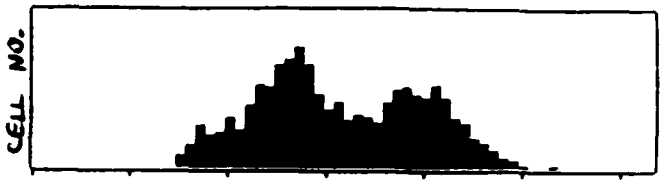
PROJECTION OF ADHERENT POPULATION ON 2 PARAMETER HISTOGRAM
GATES SET BETWEEN CHANNELS 31 TO 41

FIG 4. LIVER NON-PARENCHYMAL CELLS

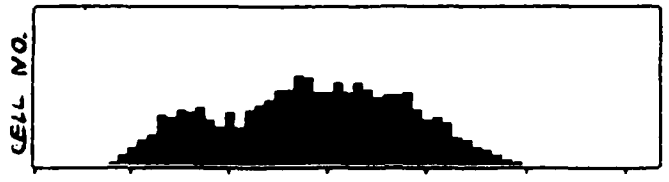
LOG RIGHT ANGLE LIGHT SCATTTER 1 PARAMETER ANALYSIS
SCALE ON ALL HISTOGRAMS IS 0 TO 64



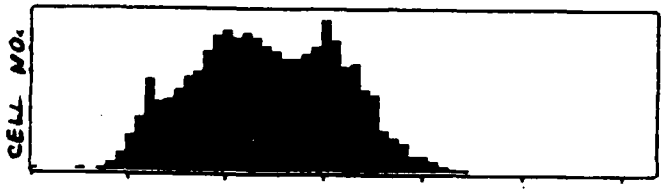
KC- 2 HR NON ADHERENT FRACTION



KC- 24 HR NON ADHERENT FRACTION

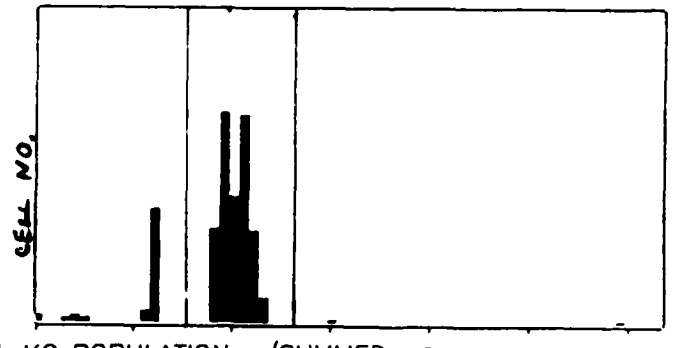


SUMMATION OF 2HR & 24 HR NON-ADHERENT FRACTION



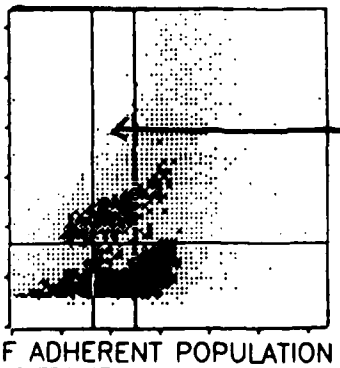
Total input NPC population

KC- TOTAL NON-ADHERED POPULATION (TIME 0)



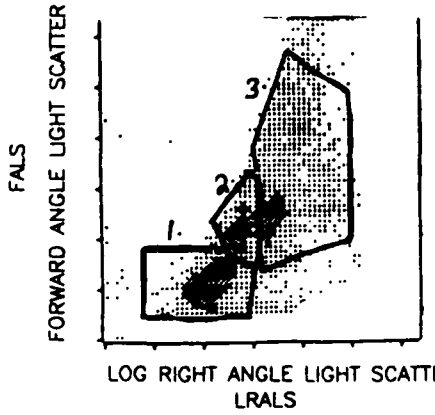
24 hour adherent NPC

TOTAL KC POPULATION - (SUMMED NON-ADHERENT FRACTIONS)
GATES SET BETWEEN CHANNELS 16 AND 25



PROJECTION OF ADHERENT POPULATION ON 2 PARAMETER HISTOGRAM
GATES SET BETWEEN CHANNELS 16 AND 25

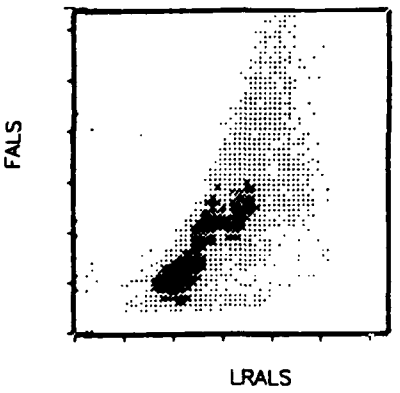
FIG. 5 2 PARAMETER ANALYSIS OF PERITONEAL CELLS



PEC – BEFORE ADHERENCE

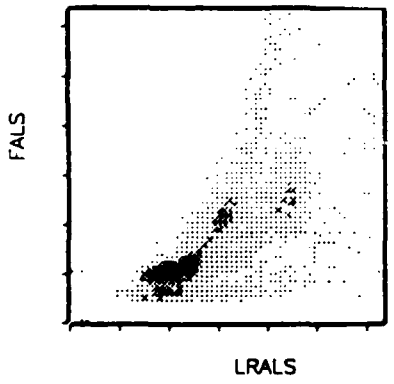
MAP 1 – LEFT POPULATION – LYMPHOCYTES
MAP 2– CENTER POPULATION
MAP 3 – RIGHT POPULATION – RESIDENT MACROPHAGES

TOTAL CELL NUMBER = 10,000



PEC – BEFORE ADHERFNCE

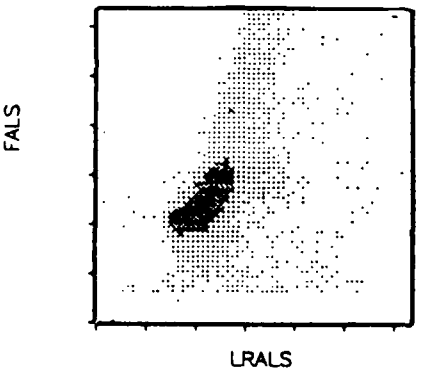
MAP 1– 53.75%
MAP 2– 14.42%
MAP 3– 26.47%



Enriched in map 1 cells

PEC – POST 2 HRS NON ADH FRACTION

MAP 1– 72.70%
MAP 2– 12.40%
MAP 3– 9.52%

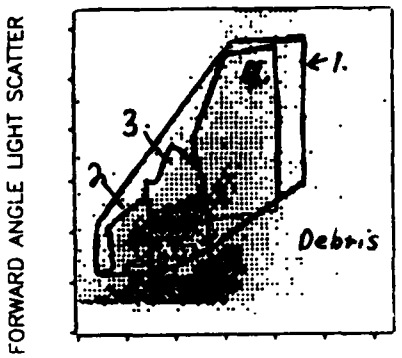


Enriched in map 2 cells

PEC NON ADH SAMPLE 24 HRS

MAP 1– 2.83%
MAP 2– 79.63%
MAP 3– 18.23%

FIG6. LIVER NON-PARENCHYMAL CELL PREPARATIONS (2 PARAMETER ANALYSIS)



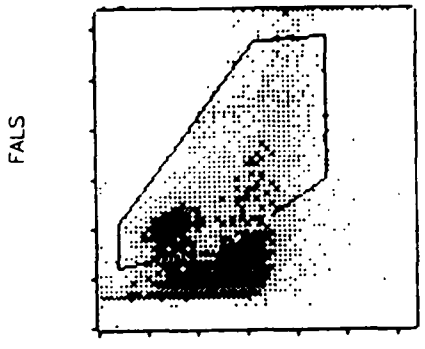
KC - BEFORE ADHERENCE

MAP 1- TOTAL CELL POPULATION EXCLUDING DEBRIS
MAP 2 - LEFT POPULATION- LYMPHOCYTES
MAP 3- CENTER POPULATION - KUPFFER CELLS
MAP 4 - RIGHT POPULATION- ENDOTHELIAL CELLS

TOTAL EVENTS IN HISTOGRAM = 10000
(INCLUDES DEBRIS)

KC - BEFORE ADHERENCE

MAP 1- 4427 TOTAL CELLS
MAP 2- 16.22%
MAP 3- 45.45%
MAP 4- 31.90%



KC - POST 2 HRS NON ADH FRACTION

MAP 1- 3592 TOTAL CELLS
MAP 2- 34.44%
MAP 3- 28.45%
MAP 4- 28.98%

KC NON ADH SAMPLE 24 HRS

MAP 1- 3003 TOTAL CELLS
MAP 2- 9.32%
MAP 3- 49.25%
MAP 4- 36.33%

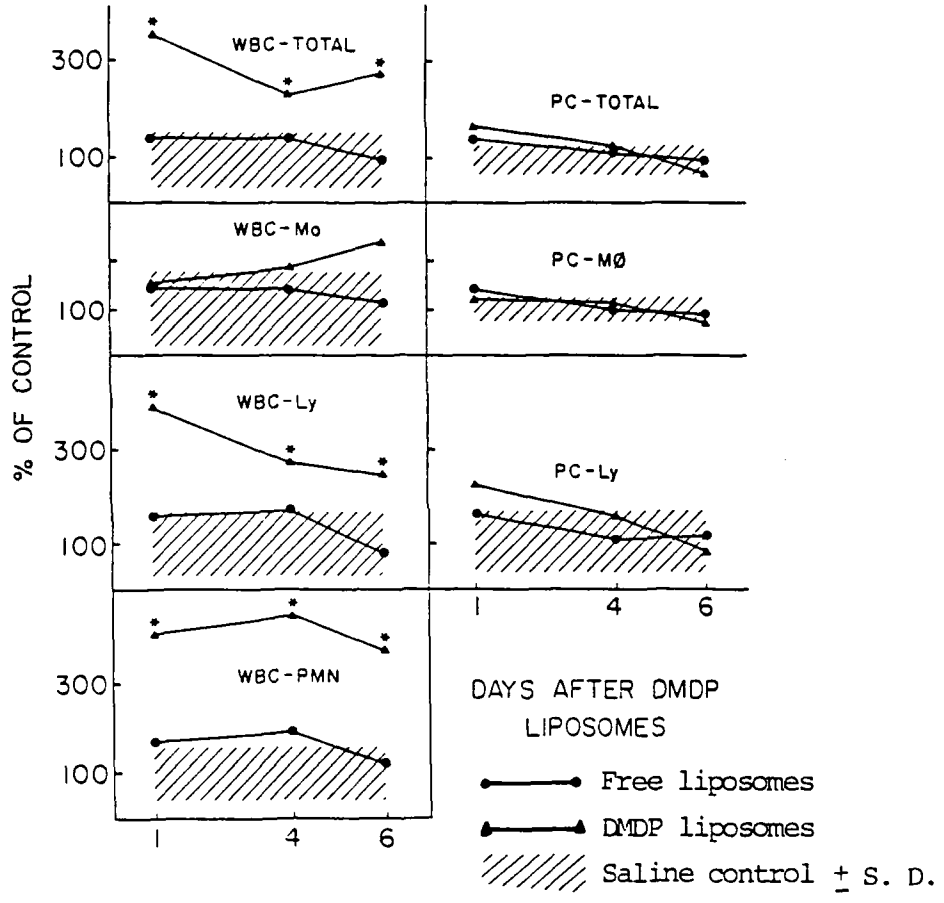


Figure 7. Effect of DMDP liposomes on peripheral white blood cell (WBC) and peritoneal cell (PC) populations. DMDP liposomes were administered i.v. on days -1 and -3 relative to the first day (day 1) on which cell populations were analyzed. Each group generally consisted of 6 mice. *Statistically significant ($p < 0.05$) as compared with saline controls.