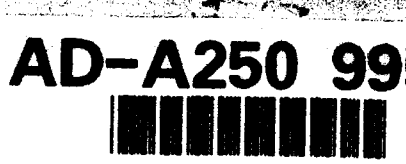

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T CELL RESPONSES TO ARENAVIRUS INFECTIONS

FINAL REPORT

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ABSTRACT

The immunological basis of the reciprocal cross-immunity induced by lymphocytic choriomeningitis virus (LCMV) and Lassa virus (LV) was investigated. A recombinant vaccinia virus that expresses the LV envelope glycoprotein precursor molecule, GP-C, (Vac-LV-G) protects C3H/HeJ mice against lethal challenge with LCMV (UBC). Protection correlated with the induction of T helper cells, but not cytotoxic T cells, that recognize LCMV antigens *in vitro*. Using synthetic peptides corresponding to potential T cell sites on LV GP-C we found that Vac-LV-G and LCMV induce a population of $CD4^+$ T cells that recognize an epitope located between residues 403-417 of LV GP-C (GP-C403-417). A synthetic peptide corresponding to these residues stimulated proliferation and IFNγ secretion by T cells primed with either virus. Five $CD4^+$ T cell clones specific for GP-C403-417 were derived from Vac-LV-G-primed mice. Of the four clones that secreted IFNγ in response to the peptide, three of them also recognized LCMV *in vitro*. Two clones (clones 9 and 11) are I-A^k-restricted and lyse target cells bearing the appropriate restriction elements in the presence of the peptide. T cell clone 9 mediates a peptide-specific delayed type hypersensitivity reaction and adoptively protects C3H/HeJ mice against lethal challenge with LCMV. These findings indicate that $CD4^+$ T cells specific for an epitope conserved between LV and LCMV GP-C are involved in the reciprocal cross-protection induced by LCMV and LV. A study of the immune response of rhesus monkeys to infection with LCMV or Vac-LV-G was begun. Monkeys primed with LCMV made a virus-specific antibody response and the PBL from one of these monkeys responded to stimulation with LCMV *in vitro*. Monkeys primed with Vac-LV-G did make an antibody response to LCMV. However, PBL that responded to LCMV *in vitro* were detected in one of three monkeys primed with Vac-LV-G. These findings indicate that primates make T cell responses to conserved epitopes on the GP-C of LV and LCMV. The epitopes these arenavirus-specific T cells recognize could not be mapped using the synthetic peptides in our collection.

FOREWORD

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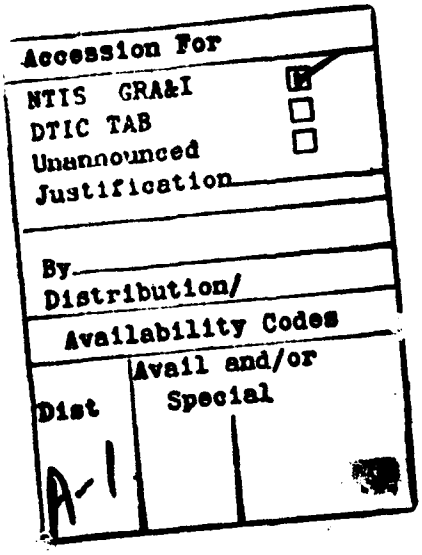

TABLE OF CONTENTS

REPORT FRONT COVER	1
REPORT DOCUMENTATION PAGE	2
ABSTRACT	3
FOREWORD	3a
TABLE OF CONTENTS	4
PREFACE	6
INTRODUCTION	7
MATERIALS AND METHODS	9
General comments.	9
Mice.	9
Monkeys.	9
Viruses.	9
Cell lines.	10
Antibodies.	10
Detection of virus-specific antibodies	11
1). Indirect immunofluorescence assays.	11
2). ELISA.	11
Peptides.	11
Immunizations.	11
1). Mice.	11
2). Monkeys.	12
Peptide-specific T cell clones.	12
T cell proliferation assays.	12
1). Murine cells.	12
2). Primate cells.	12
Stimulation of IFNγ release.	13
Secondary cytotoxic T cell induction.	13
51Cr-release cytotoxicity assays.	13
Phenotyping of T cell clones.	14
Detection of Interferon gamma.	14
Delayed-type hypersensitivity reactions.	15
Adoptive protection assays.	15
RESULTS	16
Protection against LCMV challenge by immunization with a recombinant vaccinia virus expressing LV GP-C.	16
The role of antibodies in cross-protection of Vac-LV-G-primed mice.	16
Lack of cross-reactive cytotoxic T cells in Vac-LV-G-primed mice.	17

Demonstration of cross-reactive T cells induced by Vac-LV-G by assaying lymphocyte proliferation and IFNγ secretion. 17
A virus-specific T cell epitope is located between residues 403-417 of Lassa GP-C 18
Cloned T cell lines specific for GP-C403-417 derived from Vac-LV-G-primed mice. 19
Clones 9 and 11 are I-A^k-restricted and cytotoxic for antigen-presenting cells. 20
Clone 9 mediates delayed-type hypersensitivity reactions to GP-C403-417. 20
Clone 9 T cells can protect against challenge with LCMV. 21
PRIMATE STUDIES 22
CONCLUSIONS 24
LITERATURE CITED 28
PUBLICATIONS AND MEETING ABSTRACTS 31
PERSONNEL RECEIVING PAY 31
GRADUATE DEGREES RESULTING FROM THIS CONTRACT 32
TABLES 33
TABLE 1. Synthetic peptides corresponding to segments of LV GP-C containing putative T cell epitopes. 34
TABLE 2. Immunization with Vac-LV-G protects C3H/HeJ mice against lethal LCMV challenge. 35
TABLE 3. Cross-reactive CTL are not detectable in Vac-LV-G-primed and LCMV-primed C3H/HeJ mice. 36
TABLE 4. The response of Vac-LV-G-primed and LCMV-primed lymphocytes to LCMV and the LV GP-C peptide, GP-C403-417. 37
TABLE 5. $CD4^+$ T cells respond to GP-C 403-417 38
TABLE 6. Genetic restriction and cytolytic activity of GP-C403-417 specific T cell clones. 40
TABLE 7. Protection against LCMV challenge with clone 9 T cells 42
TABLE 8. The proliferative response of PBL from monkeys primed with LCMV or Vac-LV-G to LV GP-C synthetic peptides 43
FIGURES 46
FIGURE 1. Peptide-specific T cell responses 46
FIGURE 2. GP-C403-417-specific IFNγ-secreting T cells in Vac-LV-G-primed populations can be enriched by culturing with LCMV or GP-C403-417 47
FIGURE 3. The response of GP-C403-417-specific cloned T cell lines derived from Vac-LV-G-primed mice to peptide and LCMV 48
FIGURE 4. Clone 9 T cells mediate peptide-specific DTH reaction 49
FIGURE 5. The response of PBL from rhesus monkeys immunized with LCMV or Vac-LV-G to stimulation with LCMV in vitro. 50
FIGURE 6. The effect of cell number on the response of LCMV and Vac-LV-G-primed rhesus monkey PBL to LCMV in vitro. 51

PREFACE

This report summarizes the research findings for the second half of the contract period, i.e., from February 17, 1989 to August 31, 1991. Data obtained during the first half of the contract period have been presented in the Midterm Report (report date, May 10, 1989) and where necessary we will refer to that document when discussing findings prior to Feb 17, 1989. However, some of these data will presented again in this report if they are central to the themes and conclusions of the research findings of the second half of the contract period.

INTRODUCTION

Lassa virus (LV) and lymphocytic choriomeningitis virus (LCMV) are members of the Old World (O.W.) arenavirus family. These viruses exhibit a high degree of structural homology as evidenced by their serological cross-reactivities (1) and the deduced amino sequence of their structural proteins (2). LCMV induces neurological disease in mice that is due to a cell-mediated immune mechanisms in which immunopathology and the clearance of virus are both mediated by $CD8^+$, virus-specific cytotoxic T cells (CTL) (3, 4). Whereas LCMV generally causes a mild illness in humans (1), LV causes a severe hemorrhagic disease (Lassa fever) (5) in the parts of west Africa in which it is endemic. It is estimated that there are 200,000 to 300,000 infections per year with LV which result in 3,000 to 5,000 deaths (6).

The mechanism of resistance to LV is poorly understood. There is little evidence that antibodies play a role in recovery from primary infection with LV (7). Cross-protection studies in which animals are primed with one O.W. arenavirus and challenged with another suggest that resistance to LV may be mediated by virus-specific effector T cells and that some of the T cell determinants they recognize are conserved among O.W. arenaviruses. For example, Jahrling and his colleagues showed that strain 13 guinea pigs asymptomatically infected with the Armstrong strain of LCMV (LCMV-Arm) were resistant to lethal challenge with LV (7). In addition, guinea pigs cured of LV fever by ribavirin survived lethal challenge with LCMV (WE strain) (7). Cross-protection could be transferred to normal recipients with splenic lymphocytes, but not serum, from immunized guinea pigs (7) and the protective lymphocytes were cytolytic for arenavirus-infected target cells. The pattern of cross-lysis and the transfer of cross-immunity reflected that of the cross-protection induced by virus-priming (7). In other studies, monkeys immunized with Mopeia virus, which is closely related to LV, were also resistant to otherwise lethal challenge with LV (8). Recently, cDNA encoding the LV envelope glycoprotein precursor (GP-C) (9) and the nucleocapsid (N) (10) were inserted into vaccinia virus. These recombinants, each of which expresses the product of the gene-insert upon replication, are potential vaccine candidates for LV fever. The vaccinia virus expressing LV GP-C (Vac-LV-G) can protect monkeys and guinea pigs against lethal LV challenge (9, 11).

The long term goal of the work supported by the contract was to understand the nature of the immune response to Lassa virus with particular reference to the development of a specific vaccination strategy. Specific aims include the following:

1) Evaluate the immunogenicity and protective capacity of recombinant vaccinia viruses expressing the cDNA for structural proteins of Lassa.

2) Produce a series of murine T cell lines specific for structural proteins of LCMV and Lassa virus and characterize these cell lines as to phenotype, protein specificity and functional activity *in vitro* and *in vivo*.

3) Synthesize a series of peptides corresponding to potentially protective epitopes shared between, or unique to, LCMV and Lassa viruses and use them a) to define the fine specificity of T cells which might mediate protective immunity and b) as reagents which may have potential use in immunodiagnosis.

4). Use reactive peptides as potential probes for demonstrating and measuring virus-specific T cell responsiveness of monkeys which are immune to LCMV and Lassa virus.

This report will show that Vac-LV-G will cross-immunize C3H/HeJ mice against lethal i.c. challenge with LCMV (UBC). We found that cross-protection correlated with the induction by Vac-LV-G of cross-reactive T helper/inducer cells that recognize LCMV antigens. Protection did not appear to be due to cross-reactive antibodies or cross-reactive $CD8^+$ CTL. The cross-reactive T cells were specific for a T cell epitope located between residues 403-417 of LV GP-C. A synthetic peptide corresponding to these residues (GP-C403-417) stimulated proliferation and interferon gamma (IFNγ) secretion by $CD4^+$ T cells from Vac-LV-G and LCMV primed C3H mice indicating that the epitope is conserved between LV and LCMV. A series of cloned $CD4^+$ T cell lines, specific for GP-C403-417, were derived from Vac-LV-G-primed mice. Most clones secreted IFNγ in response to peptide or LCMV. One clone, T cell clone 9, was cytotoxic *in vitro* for target cells sensitized with GP-C403-417, it mediated a peptide specific delayed type hypersensitivity (DTH) reaction and protected normal mice against lethal challenge with LCMV. These data indicate that $CD4^+$ T cells are involved in cross-protection induced by LV GP-C and that they may play a role in protection against Lassa fever.

MATERIALS AND METHODS

General comments. Unless otherwise stated, all the experiments described in this document were conducted using mice or tissues from mice. The materials and methods described below pertain to studies with mice, unless otherwise indicated.

Mice. C3H/HeJ, BALB/c, DBA/1 and C57Bl/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and used between 6 and 12 weeks of age. Recombinant inbred mice, B10.A(4R), A.TL and C3H.OH originally obtained from Jackson Laboratories were generously provided by Dr. S. Ostrand-Rosenberg (University of Maryland, Baltimore County). Unless otherwise stated in the text, experiments were conducted using C3H/HeJ mice.

Monkeys. All work with rhesus monkeys was performed at U.S.A.M.R.I.I.D. in accordance with all relevant guidelines. The designations of the monkeys, and the agents they were immunized with, are as follows:

a). 45J and 46C: unimmunized controls.

b). 4FN, 47G and 4FY: immunized with LCMV (Arm)

c). 927C, 2Y3 and 4G7: immunized with Vac-LV-G.

Monkeys were sedated with ketamine HCl inoculated intramuscularly (7mg/kg) prior to manipulation or close examination. Blood was obtained from the femoral vein using sterile Vacutainer tubes containing EDTA or heparin. The maximum volume of blood withdrawn was 5 ml per kg body weight per week.

Viruses. All viruses were stored at -70° C. The recombinant vaccinia viruses expressing the GP-C (Vac-LV-G) and nucleocapsid (Vac-LV-N) of LV has been described in detail elsewhere (9). Briefly, the genes under the control of the vaccinia P7.5 promoter were inserted into the thymidine kinase gene of the Wyeth strain of vaccinia virus. The GP-C is expressed and properly post-translationally cleaved to form the mature envelope glycoproteins GP-1 and GP-2 (9). Large stocks of Vac-LV-G and Vac-LV-N and the

parental vaccinia (Wyeth) were prepared in BSC-40 cells. Semiconfluent monolayers (70%) were infected with virus at a multiplicity of infection (MOI) = 0.1 to 1, and 2 days later cell-associated virus was obtained by harvesting the cells and freeze-thawing them three times in a small volume of medium. These preparations of viruses generally had a titer of 10^6 pfu/ml. When experiments required virus at higher concentration, band-purified Vac-LV-G was used. This was kindly prepared for us by Dr. R. Bablanian (SUNY, Brooklyn, New York) in BSC-40 cells and purified according to the method of Joklik (12). These preparations had a titer of $1x10^8$ to $3x10^8$ pfu/ml with a particle to infectivity ratio of 100:1.

The "aggressive" strain of LCMV (UBC) was obtained from Dr. C. Pfau (Rensselaer Polytechnic Institute, Troy, NY). Seed stock, which had a titer of $2x10^6$ pfu/ml when plaqued on Vero cells, was prepared by passaging the virus once in MDCK cells. Working stocks of LCMV (UBC) were prepared by infecting BHK cells with MDCK-derived virus at an MOI of 0.1. These preparations had a titer of $1x10^5$ to $2x10^6$ pfu/ml. When used to stimulate monkey PBL in culture, LCMV(UBC) was pelleted by centrifugation at 200,000 xg in a TLS-55 rotor (Beckman) using TL-100 Tabletop centrifuge. The virus pellet was resuspend to one fifth the original volume in RPMI-1640 containing 10% heat inactivated, pooled monkey serum.

The Armstrong (Arm) strain of LCMV was obtained from Dr. P.B. Jahrling (U.S.A.M.R.I.I.D., Frederick, MD) and was passaged once in mouse brains. It was stored as a 10% (w/v) mouse brain suspension in phosphate-buffered saline (PBS).

Cell lines. L cells were used as targets for *in vitro* cytotoxicity assays. The I-A^k-transfected B cell line, M12.C3-F6, and the parental line, M12.C3, which does not express cell surface MHC class II antigens (13,14) were a kind gift from Dr. S. Ostrand-Rosenberg (University of Maryland, Baltimore County).

Antibodies. A rat anti LCMV serum was used to detect LCMV antigens on infected target cells. To detect LV antigens we used the following reagents obtained from Dr. P.B. Jahrling (U.S.A.M.R.I.I.D., Frederick, MD): serum from convalescent LV-infected Rhesus monkeys, monoclonal antibodies to LV nucleoprotein (L52-93-4 BA01) and GP-2 (L52-85-6 BG). An anti vaccinia monoclonal antibody was obtained from Dr. A. Schmaljohn (U.S.A.M.R.I.I.D., Frederick, MD). Hybridoma cells secreting monoclonal antibodies to murine CD4 (GK1.5) (15), CD8 (116-13.1) (16) and to murine interferon gamma (IFNγ)

(R46A2) (17) were obtained from the American Type Culture Collection (Rockville, MD). Culture supernatant containing the XMG1.2 antibody to IFNγ (18) was a gift from Dr. B. Fox (Dept of Medicine, University of Maryland at Baltimore). The anti-CD4, anti-CD8 and anti-IFNγ antibodies were purified from spent culture supernatants by affinity chromatography on Protein G columns (Pierce, Rockford Ill) according to the manufacturers directions.

Detection of virus-specific antibodies

1). **Indirect immunofluorescence assays.** Anti-LCMV antibodies in mouse sera were measured by the indirect immunofluorescence assay (IFA) using spot slides of LCMV-infected and uninfected L cells. LV antibodies in mouse sera were assayed by IFA using spotslides of LV-infected Vero cells which were kindly provided by Dr P.B. Jahrling (U.S.A.M.R.I.I.D., Frederick, MD). Serum was diluted in PBS and reacted with the cells for 1 hour at 37^{o}. Bound antibodies were visualized by using FITC-conjugated goat anti-mouse secondary antibody and counterstaining the cells with Evans Blue (0.05% w/v).

2). **ELISA.** Monkey sera were tested for antibodies to LCMV by an ELISA using lysates of LCMV-infected cells as antigen. The assays were performed by personnel at the Rapid Diagnosis section of the Epidemiology Department at U.S.A.M.R.I.I.D.

Peptides. Synthetic peptides corresponding to segments of LV GP-C were synthesized using an Applied Biosystems 403A Peptide Synthesizer (Applied Biosystems, Foster City, CA) using the T-boc method and purified by HPLC. Their sequences are shown in Table 1.

Immunizations.

1). **Mice.** Mice were immunized intraperitoneally (i.p.) with 10^6 or 10^7 pfu of Vac-LV-G or VAC-LV-N or with 10^3 pfu of LCMV. Virus-primed mice were used no earlier than one month after immunization. For immunization with synthetic peptides, mice were injected in the rear footpads with 100 μg of peptide emulsified in 50 μl of complete Freund's adjuvant. Peptide-primed mice were used as a source of primed lymph node cells 7 to 12 days later.

2). Monkeys. Monkeys were injected subcutaneously with 10^8 pfu of Vac-LV-G per monkey in a total volume of 0.6 ml or subcutaneously with 10^3 pfu of LCMV (Arm) in a single 0.5 ml dose.

Peptide-specific T cell clones. A T cell line was established by culturing splenic lymphocytes from Vac-LV-G -primed mice ($3x10^6$ cells in 2 ml) with 1μg/ml of GP-C403-417 peptide for 7 days. The medium was replaced and the cells rested for 3 days. The T cells underwent another 5 cycles of stimulation and rest in which 10^5 responder cells per well were cultured with $3x10^6$ fresh, γ-irradiated (3000R) spleen cells and 1μg/ml of GP-C403-417. The cell line was then cloned by limiting dilution at 3, 1, 0.3 and 0.1 cells/well in flat bottom 96 well trays containing $5x10^5$ irradiated spleen cells, 1μg/ml of peptide and 10 U/ml of recombinant human IL-2 (Genzyme). Two weeks later cultures containing proliferating cells were transferred to 24 well trays and stimulated as described above with fresh stimulator cells, peptide and IL-2. Stably growing clones were restimulated every 10-14 days in this manner.

T cell proliferation assays.

1). Murine cells. T cell proliferation assays were conducted in flat bottom 96 well trays (Costar, Cambridge, MA) using RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone, Utah), glutamine, 10 mM HEPES buffer pH7.5 and $5x10^{-5}$ 2-mercaptoethanol. When splenocytes or lymph node cells were used as responders, triplicate cultures at 4×10^5 cells/well in 200μl of medium were stimulated with peptide or virus for 4 to 5 days and then pulsed with ^{3}H-thymidine for 4 hrs (1 μCi/well). When cloned T cell lines were tested, $5x10^4$ to $1x10^5$ responder cells were cultured with $5x10^5$ γ–irradiated splenocytes in the presence or absence of peptide for 2 days and then pulsed with ^{3}H-thymidine. The cells were collected using a cell harvester and incorporated radioactivity determined by liquid-scintillation counting. The results are expressed as either counts per minute (C.P.M.) incorporated into cells, or as a stimulation index (S.I.), which is defined as the ratio of the counts incorporated by cells cultured with peptide or virus to counts incorporated by cells cultured in medium alone.

2). Primate cells. Monkey peripheral blood leukocytes (PBL) were isolated from whole heparinized blood by buoyant density centrifugation on Lymphocyte Separation Medium (Organon Tecknica). The culture medium was RPMI 1640 as described above except that it was supplemented with 10% heat-

inactivated, pooled monkey serum instead of fetal bovine serum. PBL were cultured at $1x10^5$ to $4x10^5$ cells per well in round bottomed wells for 4 to 5 days with LCMV or peptides. Cell proliferation was measured as described above.

Stimulation of IFNγ release. The culture conditions employed for stimulating IFNγ release from splenocytes or cloned T cell lines were essentially the same as for the T cell proliferation assays using murine cells, the exception being that responder splenocytes were cultured at 10^6 cells/well. When LCMV was used to stimulate IFNγ release from T cell clones, $1\text{-}2x10^5$ uninduced peritoneal cells per well were cultured with $1x10^4$ pfu of LCMV for 2 days. The trays were then irradiated (1500R), half the medium removed and 10^5 responder cells added in 100µl of medium. Cultures were set up in triplicate and 100µl of supernatant was harvested at day 3 when splenocytes were tested, or on day 2 when cloned T cells were the responders. Supernatants were kept at -70° until assayed for IFNγ by ELISA.

Secondary cytotoxic T cell induction. LCMV-specific 2° CTL and vaccinia-specific 2° CTL were induced by culturing 1×10^7 splenic lymphocytes from mice primed with the appropriate virus with 10^6 pfu of LCMV or 10^6 pfu of Vac-LV-G or vaccinia respectively in 10 ml of medium. Five days later, the cultured cells were harvested, washed once, assessed for viability (>95%) and resuspended to an appropriate viable-cell concentration.

^{51}Cr-release cytotoxicity assays. To measure antiviral CTL, L cells were used as targets. They were infected with LCMV (UBC) at MOI=0.1 for 2 days or with Vac-LV-G or vaccinia for 6hrs (MOI=5) and labelled with 100-200 µCi of $Na_2\,^{51}CrO_4$. Graded number of effector cells were cultured with $1\text{-}2x10^4$ labelled target cells in 200µl of medium in round bottom wells for 6 hours at 37°. The cytotoxic activity of GP-C403-417-specific T cell clones against peptide-sensitized B cell lines was measured by culturing ^{51}Cr-labelled M12.C3 and M12.C3-F6 cells with the T cell clones in 200µl of medium for 20h in the presence or absence of 50µg/ml of peptide. At the end of the assay, 100µl of culture supernatant was harvested from the wells and the released ^{51}Cr measured in a gamma counter.

Percent cytolysis is defined as: 100 x (E-S)/(T-S)

where E, S and T are the experimental counts released, spontaneous release of label and total releasable counts, respectively. When peptides were included in the cytotoxicity assay, the spontaneous release of ^{51}Cr was determined both in the presence and in the absence of peptide.

Phenotyping of T cell clones. T cell clones were harvested 4 to 5 days after restimulation with peptide and IL-2 and and the blast cells were purified by buoyant density centrifugation on Lympholyte M (Cedarlane). They were incubated with the appropriate antibodies diluted in Dulbecco's phosphate buffered saline supplemented with 2% FBS and 0.05% sodium azide for 30 min on ice. After washing 2x they were incubated for a further 30 min with a FiTC-conjugated goat anti mouse Ig antibody, washed 2x and fixed with 2% paraformaldehyde in PBS. A minimum of 10^5 cells from each group was analyzed on an EPICS flow cytometer (Coulter).

Detection of Interferon gamma. IFNγ in culture supernatants was detected in a two-site ELISA using monoclonal antibodies to IFNγ, i.e immobilized XMG1.2 and biotinylated R46A2. The protocol is based on a previously described procedure (19). Unless otherwise stated, reagents and samples were added to the wells in a volume of 50µl and incubations were for 1 hr at room temperature. Prior to the addition of each reagent, the wells were washed at least five times with 50mM Tris-buffered saline (pH 7.5) containing 0.05% Tween 20 (TBS-Tween).

Initially, the trays were coated overnight at 4° with XMG1.2 at 1µg/ml in PBS. Nonspecific binding sites were subsequently blocked by filling the wells with a mixture of 10% FBS and 10% nonfat milk in TBS and incubating the trays for a further 2 hr at room temperature. Culture supernatants diluted in blocking solution were added to the wells and, after incubation and washing, were replaced by biotinylated R46A2 (1µg/ml in blocking solution). Subsequently, streptavidin-alkaline phosphatase diluted in TBS-Tween was added to the wells for 30 minutes. The wells were then washed at least 10 times to remove unbound streptavidin-alkaline phosphatase. Bound alkaline phosphatase was detected in a 2 step amplified reaction as described by Stanley *et al* (20). In the first step 0.0002M NADP (Sigma N-0505) in 0.05M diethanolamine buffer (pH9.5) was added to the wells. The NADP was dephosphorylated to form NAD. After 10 min the second step was initiated by adding to each well 50µl of a solution containing 3% ethanol,

2.8 U/ml diaphorase (Sigma D-2381), 45 U/ml alcohol dehydrogenase (Sigma A-3263) and the tetrazolium salt, INT violet (0.001 M) (Sigma I-8377), in 0.025M phosphate buffer (pH7.0). The NAD formed in the first step activated a redox cycle driven by alcohol dehydrogenase and diaphorase in which NAD was reduced to NADH. The NADH reduced the INT violet to form a colored product and NAD was regenerated. After 10 min the second reaction was stopped by adding 0.3M H_2SO_4 to the wells. The optical density was measured at 490 nm using a multioptic Vmax ELISA reader (Molecular Devices Corp, Menlo Park, Ca). Natural murine IFNγ (10,000 U/ml), kindly provided by Dr M. Sarzotti-Kelsoe (Veterans Administration Hospital, Baltimore), was used to generate a standard curve in all experiments. The supernatants were titrated in duplicate 5 fold serial dilutions, and those dilutions whose optical density fell in the linear portion of the standard curve were used to calculate the IFNγ content (expressed as U/ml) of the supernatants. The assay can detect 10 U/ml of IFNγ.

Delayed-type hypersensitivity reactions. The ability of a peptide-specific T cell clone to mediate a DTH response was tested by injecting into each of the two rear footpads of mice a mixture of T cells, peptide and irradiated normal spleen cells in 30μl of Hank's balanced salt solution. The thickness of the footpad was determined using a dial micrometer (Mitutoyo, Japan) immediately prior to injection of the mixture and 24 and 48hrs later. Swelling was determined as the percentage increase in footpad thickness relative to the footpad's thickness prior to injection. Each group contained at least 4 mice.

Adoptive protection assays. To measure the protective activity of T cell clones, lymphocytes were harvested 10-14 days after the last stimulation with GP-C403-417 and IL-2. The cells were washed 3 times, counted for viability and made up to a concentration of 1.33×10^8 to 6.7×10^6 viable cells/ml in Hank's balanced salts solution (HBSS). An equal volume of LCMV diluted in HBSS to twice the required concentration was added to the cell suspension. The cell/virus mixture was kept on ice until mice were injected i.c. with 30μl of the suspension. The cell dose transferred ranged from 2×10^6 to 1×10^5 cells/mouse. Mice were observed daily for a minimum of 15 days for the development of disease and deaths.

RESULTS

Protection against LCMV challenge by immunization with a recombinant vaccinia virus expressing LV GP-C. In the first half of the contract period we demonstrated the ability of Vac-LV-G to induce cross-protection against normally lethal i.c. challenge with LCMV. These data are presented once more in Table 2. C3H/HeJ mice were primed with 10^6 pfu of Vac-LV-G and challenged at least one month later with LCMV. The results, cumulated from five independent experiments, show that 89% of construct-primed mice survived challenge compared with 12% of mice primed with parental vaccinia or with 5% of unprimed controls. However, immunity was not absolute because one week after challenge Vac-LV-G primed mice showed symptoms characteristic of lymphocytic choriomeningitis. Increasing the priming dose to 10^7 pfu of Vac-LV-G did not prevent the development of symptoms after LCMV challenge, suggesting that in the previous experiment maximal priming had been achieved.

The role of antibodies in cross-protection of Vac-LV-G-primed mice. The possibility that cross-protection is mediated by cross-reactive antibodies is considered unlikely. The sera of Vac-LV-G-primed C3H mice were assayed by the indirect immunofluorescence assay (IFA) for antibodies to LV and LCMV using cells infected with either virus. The sera from mice immunized with a single dose of 10^6 or 10^7 pfu of Vac-LV-G did not specifically bind to either LV-infected or LCMV-infected cells. The sera of mice primed with two doses of 10^7 pfu of Vac-LV-G contained anti-LV antibodies, however, these sera did not specifically bind to LCMV-infected L cells (data not shown) suggesting that the construct does not induce cross-reactive antibodies. Recent reports (21,22) show that antibodies to LCMV can protect mice against a secondary challenge with LCMV and that protection is associated with the diminution of virus replication and the virtual abolition of the antiviral CTL response. As indicated in Fig. 1 of the Midterm Report, the kinetics and magnitude of the LCMV-specific CTL response of Vac-LV-G-primed C3H mice (immunized with 10^6 pfu of construct) and normal mice were virtually identical, indicating that if cross-reactive antibodies were induced by the construct, they were not present in sufficient quantities, or they were not of the correct specificity, to prevent LCMV replication and subsequent CTL induction.

Lack of cross-reactive cytotoxic T cells in Vac-LV-G-primed mice. Work from this and other laboratories established that virus-specific $CD8^+$ CTL are essential for protection against LCMV (3, 4, 23, 24). Therefore, we presumed that Vac-LV-G protected mice by inducing LV GP-C-specific CTL that recognized conserved epitopes on LCMV. Table 3 shows the result of one of several experiments designed to look for cross-reactive CTL in C3H/HeJ mice. As in other experiments (e.g., Table 5 of the Midterm Report) we conclude that there is no evidence that Vac-LV-G and LCMV primed detectable levels of reciprocally cross-reactive CTL in C3H/HeJ mice. Vac-LV-G-primed splenocytes were stimulated with LCMV for 5 days and then tested for cytolytic activity against LCMV-infected or L cells in a 5 hour ^{51}Cr-release assay. Vac-LV-G-primed splenocytes stimulated with LCMV lysed neither LCMV-infected nor Vac-LV-G-infected L cells although, under the same conditions, LCMV-primed lymphocytes readily generated 2° LCMV-specific CTL responses. Moreover, 2° LCMV-specific CTL did not lyse Vac-LV-G-infected L cells in a virus specific manner, nor could they be induced by culturing LCMV-primed splenocytes with Vac-LV-G. However, Vac-LV-G-primed lymphocytes made potent vaccinia-specific 2° CTL responses when stimulated with either Vac-LV-G or *vaccinia* virus *in vitro* , indicating that the construct was perfectly capable of stimulate $CD8^+$ T cell response against vaccinia.

Demonstration of cross-reactive T cells induced by Vac-LV-G by assaying lymphocyte proliferation and IFNγ secretion. In contrast to the results using CTL assays, cross-reactive T cells that recognized LCMV were readily demonstrable in the spleens of Vac-LV-G-primed mice by assaying the ability of lymphocytes from such mice to proliferate, or secrete IFNγ, in response to coculture with LCMV. IFNγ secretion was assayed because this cytokine appears to be important for the control of arenavirus infections (25, 26, 27). The results in Table 4 show that LCMV induced a modest but reproducible proliferative response (experiment 1A) and IFNγ secretion (experiment 2) in splenic lymphocytes from Vac-LV-G-primed mice. Clearly, the construct is less effective than LCMV in priming for responses to LCMV antigens because much stronger proliferative responses (experiment 1A) and higher levels of IFNγ secretion (experiment 2) were obtained from LCMV-primed lymphocytes stimulated with LCMV under the same conditions. Normal lymphocytes did not respond to LCMV in either assay. These data show that Vac-LV-G induces a population of cross-reactive, IFNγ-secreting T helper/inducer cells. The specificity of the cross-reactive T cells, their cytotoxic potential and their antiviral activity are described below.

A virus-specific T cell epitope is located between residues 403-417 of Lassa GP-C. As described in detail in the Midterm Report, the specificity of the T cells primed by Vac-LV-G was mapped using 11 synthetic peptides corresponding to some of the regions of LV GP-C that, according to algorithms defined by Delisi and Bersofsky (28) and Rothbard (29), are potential T cell sites. The use of these algorithms in predicting T cell sites on LV GP-C is described in the Midterm Report (page 10) and the location of potential T cell determinants on LV GP-C is also shown in Table 7 of the Midterm Report. The LV GP-C peptides, whose sequences are shown in Table 1, were initially tested for their ability to prime C3H/HeJ mice for a peptide-specific T cell response. Some peptides was also tested in BALB/c, C57Bl/6 and DBA/1 mice. Fig 1 summarizes the results of these experiments. Two peptides primed for strong proliferative responses indicating they represented T cell epitopes on LV GP-C; these were GP-C170-183 which primed C57BL/6J mice (S.I.=7), and GP-C354-368 which primed BALB/c (S.I.=19), DBA/1 (S.I.=10) and C57BL/6J mice (S.I.=7), for peptide-specific responses. The other peptides induced no response, or weak responses (S.I. ranging from 2 to 4), in at least one of the strains of mouse used in this study.

Even though several peptides contained T cell epitopes, the only "virus-specific" peptide, i.e. a peptide capable of stimulating Vac-LV-G or LCMV-primed T cells, was GP-C403-417 (amino acid sequence: IEQQADNMITEMLQK). Table 4 (experiment 1B) shows that GP-C403-417 stimulated proliferation of T cells from mice primed with either Vac-LV-G (S.I. $\geq$ 4 at 30μg/ml of peptide) or LCMV (UBC) (S.I.$\geq$ 20), but not normal mice. As shown in Table 10 of the Midterm Report, lymphocytes from mice primed with LCMV (Arm) also responded strongly to the peptide (data not shown) but not lymphocytes from mice primed with the vaccinia-LV nucleoprotein construct (data not shown). None of the other peptides stimulated Vac-LV-G or LCMV-primed lymphocytes (data not shown). These data indicate that a conserved, virus-specific T cell epitope is located between residues 403-417 of LV virus GP-C. The epitope is recognized by $CD4^+$ T helper/inducer cells because the responses of both Vac-LV-G-primed and LCMV-primed T cells to GP-C 403-417 is blocked by antibodies to CD4 but not CD8 (Table 5). LCMV-primed lymphocytes from C57BL/6J ($H\text{-}2^b$) and BALB/c ($H\text{-}2^d$) did not respond to GP-C403-417 suggesting that recognition of this epitope is probably restricted to mice of the $H\text{-}2^k$ haplotype (data not shown). In addition, they did not respond to any of the other peptides indicating that the arenavirus T cell

epitopes recognized by C57BL/6J (H-2^b) and BALB/c (H-2^d) mice primed with LCMV or Vac-LV-G are not represented in our panel of LV GP-C peptides

In addition to stimulating lymphocyte proliferation, GP-C403-417 induced IFNγ-secretion by T cells from C3H mice immunized with Vac-LV-G or LCMV (Table 4, experiment 3). As with the *in vitro* proliferative responses to LCMV, there was a greater peptide-dependent secretion of IFNγ by LCMV-primed lymphocytes (ten-fold above background) than by Vac-LV-G-primed lymphocytes (less than two-fold above background). The peptide did not stimulate IFNγ secretion from normal lymphocytes. If the cross-reactive T cells primed by Vac-LV-G are indeed specific for GP-C403-417, we reasoned that culturing Vac-LV-G-primed lymphocytes with LCMV would expand and/or induce the differentiation of peptide-specific, IFNγ-secreting T cells. The data depicted in Fig 2 supports this notion. When Vac-LV-G-primed lymphocytes that had been pre-cultured with either LCMV or GP-C403-417 for 14 days were restimulated with GP-C403-417, they secreted five to ten fold more IFNγ in response to GP-C403-417 than in response to a control peptide (GP-C456-470) or medium alone. Lymphocytes pre-cultured in the absence of virus or peptide did not secrete IFNγ above background levels when stimulated with GP-C403-417. In summary, these data clearly demonstrate that at least some of the T cells primed by Vac-LV-G are specific for an epitope(s) that resides within residues 403-417 of LV GP-C and that this epitopes is conserved on LCMV.

Cloned T cell lines specific for GP-C403-417 derived from Vac-LV-G-primed mice. To determine the role of GP-C403-417-specific T cells in cross-protection, five GP-C403-417-specific T cell clones were derived from Vac-LV-G-primed mice as described in Materials and Methods. All were Thy 1^+, $CD4^+$ and $CD8^-$ by flow cytometry (data not shown). These cloned T cell lines were tested for their ability to secrete IFNγ in response to GP-C403-417 or LCMV-infected stimulator cells. Clones 1, 3, 9 and 11, but not clone 2, secreted IFNγ when stimulated with GP-C403-417 (Fig. 3, experiments 1 and 2). No detectable IFNγ was secreted by the cloned T cells in the absence of peptide. In addition, clones 9 and 11 did not respond to stimulation by another peptide, GP-C456-470, confirming that they were peptide-specific (Fig. 3, experiments 4 and 5). Clones 1, 3, and 9 were cross-reactive for LCMV because they secreted IFNγ when cultured with LCMV-infected (but not uninfected) peritoneal cells (Fig. 3,

experiments 2 and 3). Interestingly, clone 11 which responds to GP-C403-417 does not respond to LCMV.

Clones 9 and 11 are I-A^k-restricted and cytotoxic for antigen-presenting cells. The genetic restriction of T cell clone 9 was mapped by using splenocytes from H-2 recombinant mice to present GP-C403-417. Clone 9 only responded to peptide presented by splenocytes from mice that expressed I-A^k i.e. C3H/HeJ (K^k I-A^k I-E^k D^k), B10.A(4R) (K^k I-A^k I-E^{neg} K^b) and A.TL (K^s I-A^k I-E^k D^d) mice but not splenocytes from C3H-OH (K^d I-A^d I-E^d D^k) mice (Table 6). This was confirmed, and shown to be also true for clone 11, by using a B cell line, M12.C3-F6, that is stably transfected with the α and β chain genes for I-A^k, to present the peptide (13, 14). Clones 9 and 11 secreted large amounts of IFNγ when stimulated by M12.C3-F6 in the presence of GP-C403-417, but virtually no cytokine was secreted when peptide was omitted, or when the untransfected parental cell line M12.C3 that does not express any detectable surface MHC class II molecules (13, 14) was used to present the peptide.

Microscopic examination of the cultures showed that M12.C3-F6 cells, but not parental M12.C3 cells, were killed when cultured with either clone 9 or clone 11 T cells in the presence of GP-C403-417. The cytotoxic activity of clone 9 was confirmed in a 20 hr ^{51}Cr release assay (Table 6). Clone 9 T cells lysed M12.C3-F6 target cells when GP-C403-417 was present in the culture. In the absence of peptide they were not lysed. The parental cell line M12.C3 was not killed by clone 9 either in the presence or absence of the peptide. No killing was detected at 4 hr (data not shown). GP-C403-417 did not sensitize normal L cells (H-2^k), which express only class I MHC antigens, for lysis by LCMV-specific 2° CTL (data not shown) This is consistent with the notion that this epitope is not recognized by $CD8^+$ T cells from H-2^k mice.

Clone 9 mediates delayed-type hypersensitivity reactions to GP-C403-417. A delayed type hypersensitivity (DTH) reaction to LCMV antigens that is mediated by both $CD8^+$ T cells and $CD4^+$ T cells (30, 31) is a hallmark of the immune response to LCMV. Fig. 6 shows that clone 9 T cells will mediate a local peptide-specific DTH response when transferred to normal mice. Normal C3H mice injected in the footpads with a mixture of clone 9 T cells, GP-C403-417 and irradiated normal spleen cells (added to ensure that antigen-presenting cells were not limiting) showed a peptide-specific footpad

swelling reaction 24 h later. Little or no response was seen when the peptide or clone 9 T cells were omitted from the inoculum, or an alternate peptide (GP-C456-470) was used.

Clone 9 T cells can protect against challenge with LCMV. The most interesting function of T cell clone 9 is its ability to adoptively protect C3H/HeJ mice against a low dose LCMV challenge. In the experiments presented in Table 7, clone 9 cells were admixed with a lethal dose of LCMV and injected i.c. into syngeneic and allogeneic mice. C3H mice injected with doses of clone 9 cells ranging from 2 $x10^6$ to 10^5 cells per mouse survived challenge with 20 pfu of LCMV (UBC) whereas all but one mouse injected with virus alone died. The protection is MHC-restricted since clone 9 protected only 1 of 3 BALB/c ($H\text{-}2^d$) mice against LCMV challenge and CD-1 ($H\text{-}2^q$) mice where not protected regardless of the cell dose used. Clone 9 was ineffective against a 10 fold higher challenge dose of virus.

The possibility that clone 9 T cell cultures were accidentally contaminated with LCMV so that carry-over virus or antigen immunized against a subsequent LCMV challenge was investigated, as was the possibility that LCMV was inactivated when preparing the clone 9/LCMV mixtures for i.c. inoculation. These possibility were not supported by the results of experiments designed to test these alternative explanations. LCMV could not be recovered from clone 9 T cell cultures (data not shown) and as shown in Table 8, killed clone 9 T cells were not protective, thus, making it unlikely that immunizing peptides were carried over from the T cell cultures. In addition, LCMV that was recovered from the LCMV/clone 9 mixture after one hour still killed mice (data not shown) nor were mice injected a week earlier with either clone 9 cells or culture supernatant protected against subsequent LCMV challenge (data not shown). These results argue against immunization by contaminating virus or antigen in the clone 9 T cell cultures.

PRIMATE STUDIES

The *in vitro* responses of peripheral blood lymphocytes from rhesus monkeys vaccinated with either LCMV or Vac-LV-G. Attempts were made to analyze, *in vitro,* the immune responses made by primates immunized with either LCMV (Armstrong) or Vac-LV-G. In collaboration with Dr. C. J. Peters and Dr. P. B. Jahrling at U.S.A.M.R.I.I.D., rhesus monkeys were immunized with the above viruses. Three monkeys were immunized with 10^8 pfu of Vac-LV-G intradermally, three monkeys with 10^3 pfu of LCMV (Arm) and two monkeys served as unimmunized controls. The sera from these monkeys were tested 1 year later for antibodies to LCMV by ELISA. All three LCMV-primed monkeys had detectable serum antibodies to LCMV; none of the sera from Vac-LV-G-primed monkeys or normal monkeys had antibodies that cross-reacted with LCMV (data not shown).

At various times after immunization PBL from these monkeys were tested for reactivity to LCMV using *in vitro* lymphocyte proliferation assays. The results of one such experiment conducted at day 145 post-immunization is shown in Fig 5. Virus-specific, dose-dependent lymphocyte proliferation was demonstrated in one of three LCMV-primed monkeys (4FY) and one of three Vac-LV-G-primed monkeys (927C), but not in unimmunized monkeys, indicating that it is possible to demonstrate arenavirus-specific T cell responses in primates. The PBL from all monkeys responded to mitogenic stimulation with Con A (data not shown), indicating that the culture conditions could support the proliferation of primate PBL. However, the fact that LCMV stimulated the PBL from only one of three three LCMV-primed monkeys may indicate that the conditions used for detecting LCMV-specific T cells were not optimal. The reactivity of PBL from monkeys 4FY and 927C for LCMV was confirmed in a subsequent experiment Fig. 6 in which the number of responding cells was titrated and proliferation measured on days 4 and 5. The results show that at least $2x10^5$ cells/well are required in order to observe virus-specific proliferation. Proliferation in response to LCMV was observed for 4FY and 927C PBL on day 5 although the response of 927C PBL was detectable on day 4.

Attempts were made to map the epitope recognized by the T cells from monkeys 4FY and 927C using 5 peptides that spanned the region from residues 383 to 417 and residues 446 to 470 of LV GP-C. These were the LV GP-C peptides in our panel that had the highest homology with the corresponding segment from LCMV GP-C (Midterm Report, Table 8). None of these peptides stimulated PBL from either

monkey 4FY or 927C even though their PBL responded to LCMV (Table 8). These data suggest that the epitope(s) recognized by monkey GP-C-specific T cells were not represented by these peptides.

CONCLUSIONS

Our knowledge of the immune response to arenaviruses is largely based on studies of LCMV infection in mice. The role of virus-specific $CD8^+$ CTL in immunity to LCMV and the pathology of LCM disease is now well established (3, 4, 23, 24) and some of the epitopes they recognize on the GP-C and nucleoprotein of LCMV have been defined (32, 32a, 33, 34). Little is known of the specificity of $CD4^+$ T cells in these responses and the nature of the immune response to other arenaviruses such as LV.

LCMV and LV can induce reciprocal cross-protective immunity in rodents and primates (7). Our finding that C3H/HeJ mice primed with Vac-LV-G are protected against an otherwise lethal challenge with LCMV clearly demonstrates that cross-protective epitopes reside on LV GP-C. These data extend previous reports that vaccinia-vectored LV GP-C protects guinea pigs and monkeys against lethal LV challenge (9,11). Hany *et al* (35) reported that $H\text{-}2^k$ mice primed with a vaccinia construct that expresses LCMV GP-C showed a variable and low degree of protection against LCMV (WE) in virus clearance assays early, but not late, after immunization. However, in contrast to our results, $H\text{-}2^k$ mice were not protect against lethal i.c. challenge with LCMV. The conflicting results may reflect differences in the challenge doses and strains of mice and LCMV used in our respective studies.

Vac-LV-G-primed lymphocytes proliferate and secrete IFNγ when stimulated with LCMV *in vitro*, thus, supporting the hypothesis that Vac-LV-G confers protection against LCMV challenge by priming T cells that are specific for epitopes common to the envelope glycoproteins of LV and LCMV. The *in vitro* experiments utilizing rhesus monkey PBL showed that arenavirus-primed lymphocytes can also be demonstrated in primates immunized with LCMV or Vac-LV-G. The proliferative response of PBL from at least one Vac-LV-G-primed monkey to LCMV supports the notion that primates recognize cross-reactive determinants on the GP-C of LCMV and LV. Immunization of monkeys or mice with Vac-LV-G did not induce detectable antibodies to LCMV suggesting that cross-protection in our model is not mediated by antibodies. This is consistent with previous observations that cross-protection does not correlate with the presence of cross-neutralizing antibodies (7).

Given the central role that $CD8^+$ T cells play in immunity to LCMV (3, 4, 23, 24) it was surprizing to find that cross-protection did not correlate with the induction of cross-reactive CTL. Reciprocally cross-reactive CTL were not primed in C3H mice by immunization with LCMV or Vac-LV-G. LCMV-specific 2° CTL were not induced either when Vac-LV-G-primed lymphocytes were stimulated with LCMV or when LCMV-

primed lymphocytes were stimulated with Vac-LV-G. Moreover, our attempts at inducing cross-reactive CTL that recognized both LFV and LCMV GP-C by priming C3H mice with Vac-LV-G and then repeatedly stimulating their T cells in culture with LCMV invariably led to the generation of non-specific T cell lines (La Posta and Cole, unpublished). It should be noted that the same approach was successfully used in the past to generate LCMV-specific CTL from LCMV-immune mice (3). There are two possible reasons why cross-reactive H-2^k-restricted CTL lines could not be prepared. First, C3H mice may be poor responders to LFV GP-C since mice of the H-2^k haplotype appear to make little or no CTL response to LCMV GP-C (35, 36). Alternatively, H-2^k-restricted CTL may recognize sequences on LFV GP-C that are not conserved on LCMV GP-C and, therefore, cannot be stimulated by LCMV. Type-specific $CD8^+$ CTL responses for different strains of LCMV have been reported and a clonal analysis of these responses indicates that a single amino acid difference in a T cell epitope between two strains can result in a lack of cross-reactivity (36a).

The specificity of the arenavirus-specific T cells induced by Vac-LV-G was tested using synthetic peptides corresponding to eleven potential T cell sites on LV GP-C. Of these, one peptide corresponding to residues 403-417 of LV GP-C (IEQQADNMITEMLQK) stimulated $CD4^+$ T cells from Vac-LV-G and LCMV-primed C3H mice to proliferate. Several of the other peptides e.g., GP-C170-183 and GP-C354-368 induced peptide-specific T cell and antibody responses but these peptides were not recognized by LCMV or Vac-LV-G-primed T cells. This is the first description of a "virus-specific" epitope on LV and LCMV GP-C that is recognized by murine $CD4^+$ T cells. The epitope is distinct from the H-2^b MHC class I-restricted CTL determinants on LCMV GP-C (residues 34-40: AVYNFAT, and residues 278-286: VENPGGYCL) (32, 32a, 33, 34). Why the immune system selects GP-C403-417 over the other potential epitopes during virus infection is not known, but it may relate to the efficiency with which the epitope binds MHC class II molecules and/or possibly the presence of residues outside the sites that may affect degradation and presentation (reviewed in 37). Similar attempts using peptides to map the epitopes recognized by LCMV- or Vac-LV-G-immune monkey T cells were not successful. This suggests that the epitopes they do recognize are not represented among the peptides tested and that peptides corresponding to other T cell epitopes on LV GP-C should be synthesized and tested.

$CD4^+$ T cell clones specific for GP-C403-417 were established from Vac-LV-G-primed mice. Three of the five T cell clones (clones 1, 3, and 9) may be classified as Type 1 T helper (Th1) cells because they

secrete IFNγ (38) when stimulated by specific peptide or LCMV-infected peritoneal cells. Clone 11 also secretes IFNγ, however, it does not respond to LCMV-infected peritoneal cells. This suggests that there may be a difference in the fine specificity of T cell clone 11, i.e., the critical residues on GP-C403-417 that clone 11 recognizes may not be conserved on LCMV (UBC) GP-C.

Clones 9 and 11 are I-A^k-restricted and both are cytolytic for I-A^k-bearing cells in the presence of GP-C403-417. These results indicate that virus-specific, IFNγ-secreting $CD4^+$ T cells can lyse MHC class II-bearing target cells expressing an arenaviral epitope(s). Cytotoxic class II-restricted T cells have been described in other viral infections including influenza virus (39), vesicular stomatitis virus (40) and measles virus (41). Clone 9 also mediates a peptide-specific local DTH reaction when transferred to normal mice. This observation is in keeping with previous reports that Th1 cells mediate DTH reactions (42). Since DTH responses to LCMV involve $CD4^+$ T cells (30, 31), it suggests that some of $CD4^+$ T cells that mediate DTH reactions to LCMV in mice of the H-2^k haplotype are specific for GP-C403-417.

T cell clone 9 protects C3H mice against lethal LCMV challenge with a low dose of LCMV. This is the first demonstration that a $CD4^+$ T cell clone specific for an epitope on GP-C can protect against LCMV. This finding indicates that $CD4^+$ T cells, while not absolutely necessary for protection against LCMV (23, 24), do have antiviral activity and it supports the notion that the cross-protection engendered by immunization with Vac-LV-G can be mediated by cross-reactive $CD4^+$ T cells. Earlier evidence indicating a role for $CD4^+$ T cells in immunity to LCMV include the reduction of CTL responses to LCMV (43) in mice treated with anti $CD4^+$ antibodies and a diminished clearance of LCMV from the footpads of mice treated with anti $CD4^+$ (44) or transfused with virus-specific lymphocytes depleted of $CD4^+$ T cells (44). In other experimental virus infections $CD4^+$ T cells have been shown to prevent neurological disease caused by coronaviruses (45) although clearance of the virus required both $CD4^+$ and $CD8^+$ T cell subsets (45, 46). In addition, $CD4^+$ T cells are involved in the pathology of Borna disease (47).

T cell clone 9 may effect its protective activity by (a) secreting antiviral cytokines such as IFNγ, (b) lysing virus-infected MHC class II-bearing cells, or (c) providing help for a more rapid induction of virus-specific $CD8^+$ MHC class I-restricted CTL (48). IFNγ has been implicated in immune effector mechanisms against viruses (49), intracellular bacteria (50) and protozoan parasites (51). In addition to its antiviral activity, IFNγ is a costimulator of $CD8^+$CTL (52), and is also secreted by them (53). Moreover, IFNγ induces or augments the expression of MHC class I (54) and class II (55) cell surface molecules that are

necessary for antigen recognition by $CD8^+$ (56) and $CD4^+$ (57) effector T cells respectively. Of direct relevance to our studies is the demonstration that LCMV can be cleared from mice by the administration of IFNγ (27). When injected into immunocompetent mice acutely infected with LCMV, antibodies which neutralize IFNγ can prevent virus clearance (25, 26) and the development of lethal CNS disease (25, 26). Antibodies to IFNγ may inhibit immunopathology by neutralizing the IFNγ secreted by CTL or by blocking the induction of such CTL (25) since IFNγ is a cofactor in CTL induction and differentiation (52). These data point to the possibility that the IFNγ secreted by T cell clone 9 is responsible for its antiviral activity.

The role of $CD8^+$ T cells in the resistance of Vac-LV-G-primed C3H mice to LCMV challenge remains enigmatic. Neither LCMV-specific $CD8^+$ CTL, nor their primed precursors, are detectable in Vac-LV-G-primed mice prior to challenge but both are readily detectable 7 days after LCMV challenge. The CTL response appears to be essentially a primary response since the kinetics of induction and the peak virus-specific cytotoxic activity of splenocytes from Vac-LV-G-primed mice are similar to that of normal mice similarly challenged with LCMV. However, we cannot exclude the possibility that cross-reactive $CD8^+$ T cells were present at extremely low frequencies or that cross-protection is due to both cross-reactive $CD4^+$ and $CD8^+$ T cells.

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PUBLICATIONS AND MEETING ABSTRACTS

PUBLICATIONS:

1991: Vincent J. La Posta, David D. Auperin, and Gerald A. Cole

CROSS-PROTECTION AGAINST LYMPHOCYTIC CHORIOMENINGITIS VIRUS BY A CLONED CD4+ T CELL LINE SPECIFIC FOR AN EPITOPE ON LASSA VIRUS ENVELOPE GLYCOPROTEIN.

Submitted for publication to The Journal of Virology.

MEETING ABSTRACTS:

1991: La Posta V. J. and Cole G.A. **A conserved epitope on GP-2 of Lassa and LCM viruses recognized by cross-protective CD4+ T cells.** (Oral presentation). 10th Annual Meeting of the American Society for Virology. Ft Collins, Colorado.

1990: La Posta V.J. and Cole G.A. **T cell determinants on Lassa virus glyco-protein (GP-C).** (Oral presentation/Abstract# W3-007) VIIIth International Congress of Virology. Berlin.

1989: La Posta V.J. and Cole G.A. **T cell determinants on Lassa virus glycoprotein.** (Oral presentation). Annual Meeting of the American Society for Tropical Health and Medicine. Hawaii.

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GRADUATE DEGREES RESULTING FROM THIS CONTRACT:

No degrees resulted from contract support.

TABLES

TABLE 1. Synthetic peptides corresponding to segments of LV GP-C containing putative T cell epitopes.

RESIDUE NUMBERS	PEPTIDE SEQUENCE
75-88	METLNMTMPLSCT
170-183	HSYAGDAANHCG
308-320	EFCDMLRLFDFNK
354-368	HLRDIMGIPYCNYSK
383-397	PKCWLVSNGSYLNET
393-407	YLNETHFSDDIEQQA
403-417	IEQQADNMITEMLQK
410-425	MITEMLQKEYMERQGK
436-450	FSTSFYLISIFLHLV
446-460	FLHLVKIPTHRHIVG
456-470	RHIVGKSCPKPHRLN

TABLE 2. Immunization with Vac-LV-G protects C3H/HeJ mice against lethal LCMV challenge.

IMMUNIZING VIRUS	SURVIVORS/TOTAL	%SURVIVORS
Vac-LV-G	24/27	89
Vaccinia	2/17	12
NONE	1/21	5
LCMV	10/10	100

C3H/HeJ mice were primed ip with either 10^6 pfu of Vac-LV-G or vaccinia virus or 10^3 pfu of LCMV then challenged i.c. at least 1 month later with $2x10^2$ pfu of LCMV (UBC). These results were pooled from 4 independent experiments. NONE vs Vac-LV-G, $p < 0.0005$; Vac-LV-G vs VACCINIA $p < 0.0005$; NONE vs VACCINIA $p > 0.05$; LCMV vs Vac-LV-G $p > 0.05$

TABLE 3. Cross-reactive CTL are not detectable in Vac-LV-G-primed and LCMV-primed C3H/HeJ mice.

			% lysis of L cells infected with:			
Priming virus	Virus *in vitro*	E:T	Nothing	LCMV	Vac-LV-G	Vaccinia
Vac-LV-G	LCMV	15	1	1	22	15
		5	-1	0	8	5
"	Vac-LV-G	5	18	15	87	58
		2	2	1	50	36
"	Vaccinia	5	17	14	86	75
		2	4	4	64	57
"	Medium	15	1	2	13	10
LCMV	LCMV	15	17	62	20	19
		5	3	20	3	5
		2	2	9	2	1
"	Vac-LV-G	15	6	6	6	8
"	Vaccinia	15	2	10	6	7
"	Medium	15	15	22	15	23

Splenocytes from C3H/HeJ mice immunized with Vac-LV-G (twice with 10^7 pfu one month apart) or LCMV were stimulated in separate cultures with either LCMV, Vac-LV-G, vaccinia virus or medium alone for 5 days and the cells tested for cytotoxic activity against normal L cells and L cells infected with LCMV, Vac-LV-G or vaccinia virus in a 5 hr ^{51}Cr release assay. The target cells were shown by IFA, using the appropriate antibodies, to be greater than 90% infected with LCMV (rat anti LCMV serum), 70% infected with Vac-LV-G (rhesus monkey anti Lassa virus serum and anti vaccinia monoclonal antibody) and 90 % infected with vaccinia (monoclonal anti vaccinia antibody).

TABLE 4. The response of Vac-LV-G-primed and LCMV-primed lymphocytes to LCMV and the LV GP-C peptide, GP-C403-417.

EXPT	ASSAY	PEPTIDE (µg/ml)	LCMV (pfu/well)	IMMUNIZING VIRUS				
				Vac-LV-G #1	Vac-LV-G #2	Vac-LV-G #3	LCMV	NONE
1A[a]	Lymphocyte Proliferation		10^5	6770	2638	8371	30178	1721
			$5x10^3$	6038	3992	5957	35076	1672
			0	2219	990	2263	966	1172
1B[a]	Lymphocyte Proliferation	30		7650	4840	7040	32186	1446
		10		7769	3979	6457	31442	962
		3		6699	3251	5282	27946	1101
		1		3848	2990	4022	17231	609
		0		1815	984	1560	961	1362
						Vac-LV-G	LCMV	NONE
2[b]	IFNγ secretion		$3x10^4$			335	539	7
			0			191	59	8
						Vac-LV-G	LCMV	NONE
3[c]	IFNγ secretion	10				374	549	12
		0				191	41	4

a: The proliferative response of splenic lymphocytes from mice immunized with either Vac-LV-G, LCMV or nothing one month earlier and stimulated with graded concentrations of LCMV or peptide GP-C403-417. Thymidine incorporation was measured on day 4 and is expressed as C.P.M. In this experiment the lymphocytes from three individual Vac-LV-G-primed mice were tested separately.

b: IFNγ secretion induced by LCMV from splenic lymphocytes of mice immunized with either, Vac-LV-G, LCMV or nothing at least one month earlier. Culture supernatants were harvested on day 4 and the IFNγ measured by ELISA. The results are the average of three experiments and are expressed as U/ml.

c: IFNγ secretion induced by peptide GP-C403-417 from splenic lymphocytes of mice immunized with either, Vac-LV-G, LCMV or nothing at least one month earlier. Culture supernatants were harvested on day 4 and the IFNγ measured by ELISA. The results are the average of three experiments and are expressed as U/ml.

TABLE 5. CD4+ T cells respond to GP-C 403-417[a]

	^{3}H-thymidine incorporated (C.P.M.)		
	Experiment 1	Experiment 2	
Ab (μg/ml)	LCMV-primed	Vac-LV-G-primed	
		mouse 1	mouse 2
Peptide only (no antibody)	11,306	10,415	17,782
medium only (no antibody)	1,247	4,791	8,018
anti-CD4 (10)	666	1,791	4,276
anti-CD4 (1)	2,545	N.D.[b]	N.D.
anti-CD8 (10)	8,449	10,839	15,146
anti-CD8 (1)	12,068	N.D.	N.D.

a Splenic lymphocytes from LCMV-primed mice (a pool of cells from 2 mice) and Vac-LV-G-primed mice (cells from 2 mice tested individually) were tested in two separate experiments. They were cultured for 5 days with GP-C 403-417 (10μg/ml) in the absence or presence of anti CD4 (GK1.5) or anti CD8 (116-13.1) at concentrations indicated above.

b Not done.

BLANK

TABLE 6. Genetic restriction and cytolytic activity of GP-C403-417 specific T cell clones.

Experiment	T cell clone	Stimulator cell (or target cell)	Haplotype K I-A I-E D	Assay
				IFNγ (U/ml)
1 [a]	9	C3H/HeJ	k k k k	1328
		A.TL	s k k d	660
		B10.A(4R)	k k neg b	290
		C3H.OH	d d d k	<LOD [b]
				IFNγ (U/ml)
2 [c]	9	M12.C3-F6	d k neg d	20,000
		M12.C3	d neg neg d	226
	11	M12.C3-F6	d k neg d	12,400
		M12.C3	d neg neg d	<LOD
				Lysis (%)
3 [d]	9	^{51}Cr M12.C3-F12 plus peptide	d k neg d	52.4
		^{51}Cr M12.C3-F12 medium control	d k neg d	4.3
		^{51}Cr M12.C3 plus peptide	d neg neg d	5.0
		^{51}Cr M12.C3 medium control	d neg neg d	7.0

a 10^5 clone 9 T cells were stimulated with 50μg/ml of GP-C403-417 in the presence of $5x10^5$ γ-irradiated splenocytes. In the absence of peptide no detectable IFNγ was secreted.

b Below the limit of detection (LOD), which in experiments 1 and 2 were 75 U/ml and 200U/ml respectively.

c B cell lines M12.C3-F6 and M12.C3 were cultured at 10^5 cells/well with 10^5 clone 9 or clone 11 T cells in the presence or absence of 50μg/ml of GP-C403-417. In the absence of peptide no detectable IFNγ was secreted.

d $2x10^4$ ^{51}Cr-labelled target cells were cultured with $2x10^5$ clone 9 T cells for 20hrs in the presence or absence of 50µg/ml of GP-C403-417. The spontaneous release of label from each target was <25% in the presence or absence of peptide.

TABLE 7. Protection against LCMV challenge with clone 9 T cells[a].

EXPT	CELL DOSE	CHALLENGE DOSE (pfu)	SURVIVORS/TOTAL C3H/HeJ	Allogeneic
1	$1x10^6$	20	5/5	N.D[b].
	NONE		1/5	N.D.
				BALB/C (H-2^d)
2	$2x10^6$	20	5/5	1/3
	NONE		0/5	0/3
	$2x10^6$	200	0/5	N.D.
	NONE		0/5	N.D.
				CD-1 (H-2^q)
3	$1x10^6$	20	4/5	0/6
	$3x10^5$		4/5	0/6
	$1x10^5$		4/4	0/6
	NONE		0/5	0/6
	10^6 killed[c]		0/5	0/6

a: The indicated amounts of LCMV and clone 9 T cells were injected i.c. as a mixture into mice in 30µl of Hank's balanced salt solution. The T cells were used 10-14 days after stimulation. In experiment 3 dead cells and debris were removed by centrifugation on a Lympholyte M gradient.

b: Not done.

c: Frozen and thawed three times.

Table 8. The proliferative response of PBL from monkeys primed with LCMV or Vac-LV-G to LV GP-C synthetic peptides[a].

	Monkey designations and immunizing virus			
	4FY	927C	46C	45J
Antigen in vitro	LCMV	Vac-LV-G	Unimmunized	Unimmunized
Medium alone	912	3443	408	307
LCMV	10,341	9,152	1,004	422
GP-C383-397	1,049	2,577	599	234
GP-C393-407	599	4,720	346	254
GP-C403-417	365	4,260	289	478
GP-C446-440	456	2,171	714	200
GP-C456-470	1,051	2,619	321	261

[a]: $2x10^5$ purified PBL were cultured for 5 days with LCMV or LV GP-C peptides at 50µg/ml

FIGURE LEGENDS

FIGURE. 1. Peptide-specific T cell responses[a].

[a]: Peptides encompassing residues 75-88, 170-183, 308-320, 354-368, and 410-425 of LV GP-C were tested in C3H/HeJ (H-2^k), C57BL/6J (H-2^b), BALB/cJ (H-2^d) and DBA/1J (H-2^q) mice in 5 separate experiments (one experiment for each peptide). Peptides 383-397, 393-407, 403-417 and 446-460 were all tested in one experiment using C3H/HeJ mice. Mice were immunized with 100 µg of peptide emulsified in complete Freund's adjuvant and draining lymph node cells tested 7-12 days latter.

FIGURE. 2. GP-C403-417-specific IFNγ-secreting T cells in Vac-LV-G-primed populations can be enriched by culturing with LCMV or GP-C403-417[a].

[a]: Lymphocytes from mice immunized 1 month earlier with Vac-LV-G were cultured with medium alone, LCMV (10^5 pfu/well) or GP-C403-417 (10µg/ml) in 24 well trays for 5 days. The medium was replaced and the cells cultured for a further 9 days. The cells were then harvested and stimulated in 96 well plates for 2 days at $5x10^4$ viable cells/well with medium, GP-C403-417 (10µg/ml) or another peptide GP-C456-470 (10µg/ml) in the presence of fresh, irradiated spleen cells ($5x10^5$/well). IFNγ in the supernatants was measured by ELISA.

FIGURE 3. The response of GP-C403-417-specific cloned T cell lines derived from Vac-LV-G-primed mice to peptide and LCMV.[a]

[a]: Cloned T cell lines were stimulated with the peptides GP-C403-417 or GP-C456-470 (irrelevant peptide) (5-10 µg/ml) in the presence of irradiated syngeneic splenocytes or with LCMV-infected peritoneal cells. The clones were used 10 to 14 days after last stimulation, except in experiment 2 where clones 9 and 11 were tested at day 8. In experiments 1 to 4 IFNγ secretion was assayed whereas in experiment 5 the assay

was the proliferation of clone 11 T cells. The horizontal bars indicate the limits of detection for IFNγ in the various experiments. No detectable IFNγ was secreted by the T cell clones in the absence of peptide, nor by splenocytes (with or without peptide) or by peritoneal cells (normal or LCMV-infected) (data not shown).

FIGURE 4. Clone 9 T cells mediate peptide-specific DTH reaction[a].

[a]: Normal C3H/HeJ mice were injected in the footpad with combinations of 10^6 clone 9 T cells, 4μg peptide (GP-C403-417 or GP-C456-470) and $2x10^6$ irradiated (1500 R) normal splenocytes in a volume of 30μl. Footpad thickness was measured immediately before injection and 24 h later. Swelling is expressed as the % increase in thickness. Shown are the results from 2 experiments.

FIGURE 5. The response of PBL from rhesus monkeys immunized with LCMV or Vac-LV-G to stimulation with LCMV *in vitro*.[a]

[a]: 4×10^5 purified PBL from monkeys immunized with either LCMV (Arm), Vac-LV-G or nothing were cultured with two doses of LCMV (UBC) or medium alone for 4 days.

FIGURE 6. The effect of cell number on the response of LCMV and Vac-LV-G-primed rhesus monkey PBL to LCMV *in vitro*.[a]

[a]: Monkey PBL were cultured at 1×10^5 and 2×10^5 cells/well with LCMV or medium alone for 4 days or 5 days.

FIGURE 1.

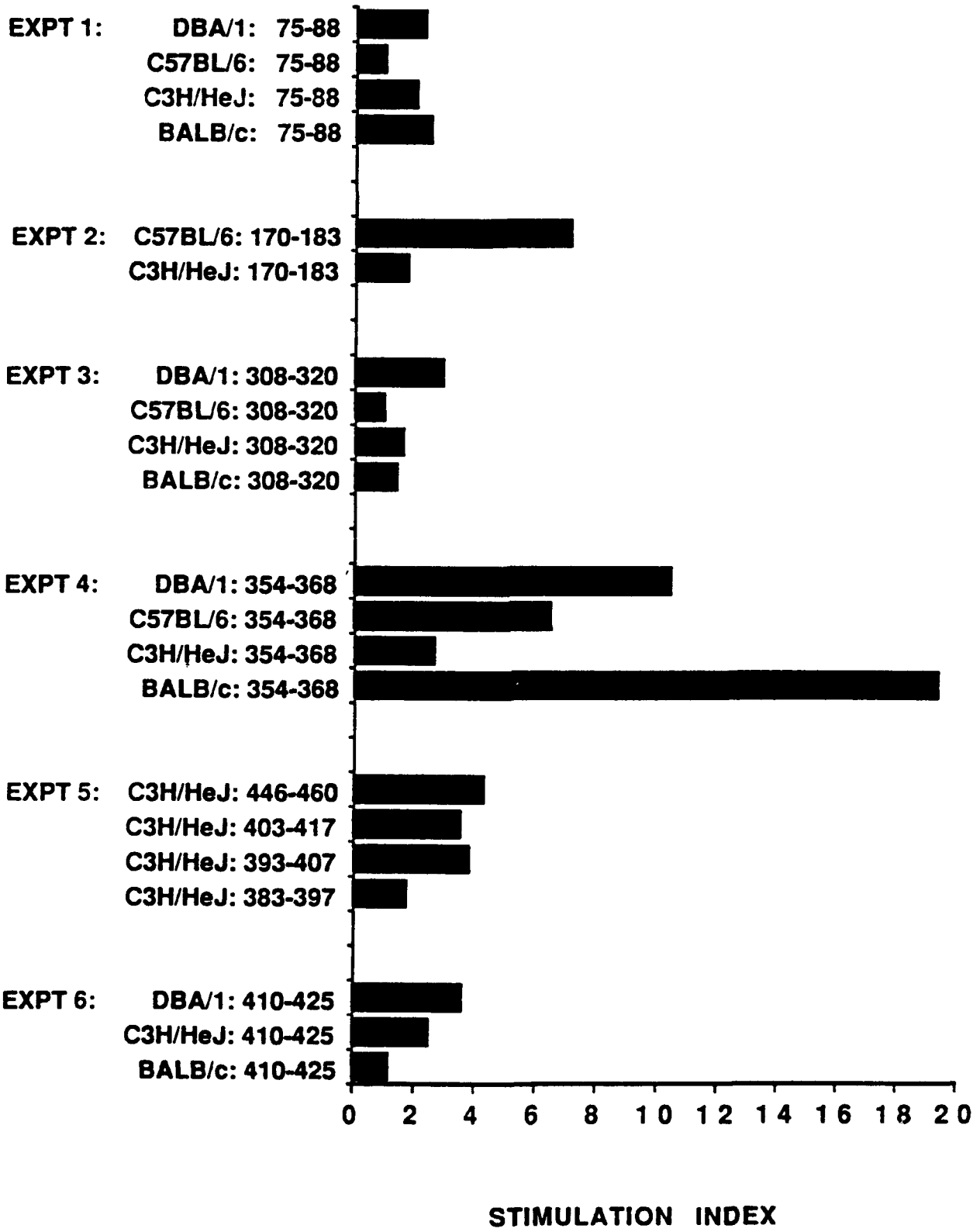

EXPT 1:
DBA/1: 75-88
C57BL/6: 75-88
C3H/HeJ: 75-88
BALB/c: 75-88
EXPT 2:
C57BL/6: 170-183
C3H/HeJ: 170-183
EXPT 3:
DBA/1: 308-320
C57BL/6: 308-320
C3H/HeJ: 308-320
BALB/c: 308-320
EXPT 4:
DBA/1: 354-368
C57BL/6: 354-368
C3H/HeJ: 354-368
BALB/c: 354-368
EXPT 5:
C3H/HeJ: 446-460
C3H/HeJ: 403-417
C3H/HeJ: 393-407
C3H/HeJ: 383-397
EXPT 6:
DBA/1: 410-425
C3H/HeJ: 410-425
BALB/c: 410-425
0 2 4 6 8 10 12 14 16 18 20
STIMULATION INDEX

FIGURE 2.

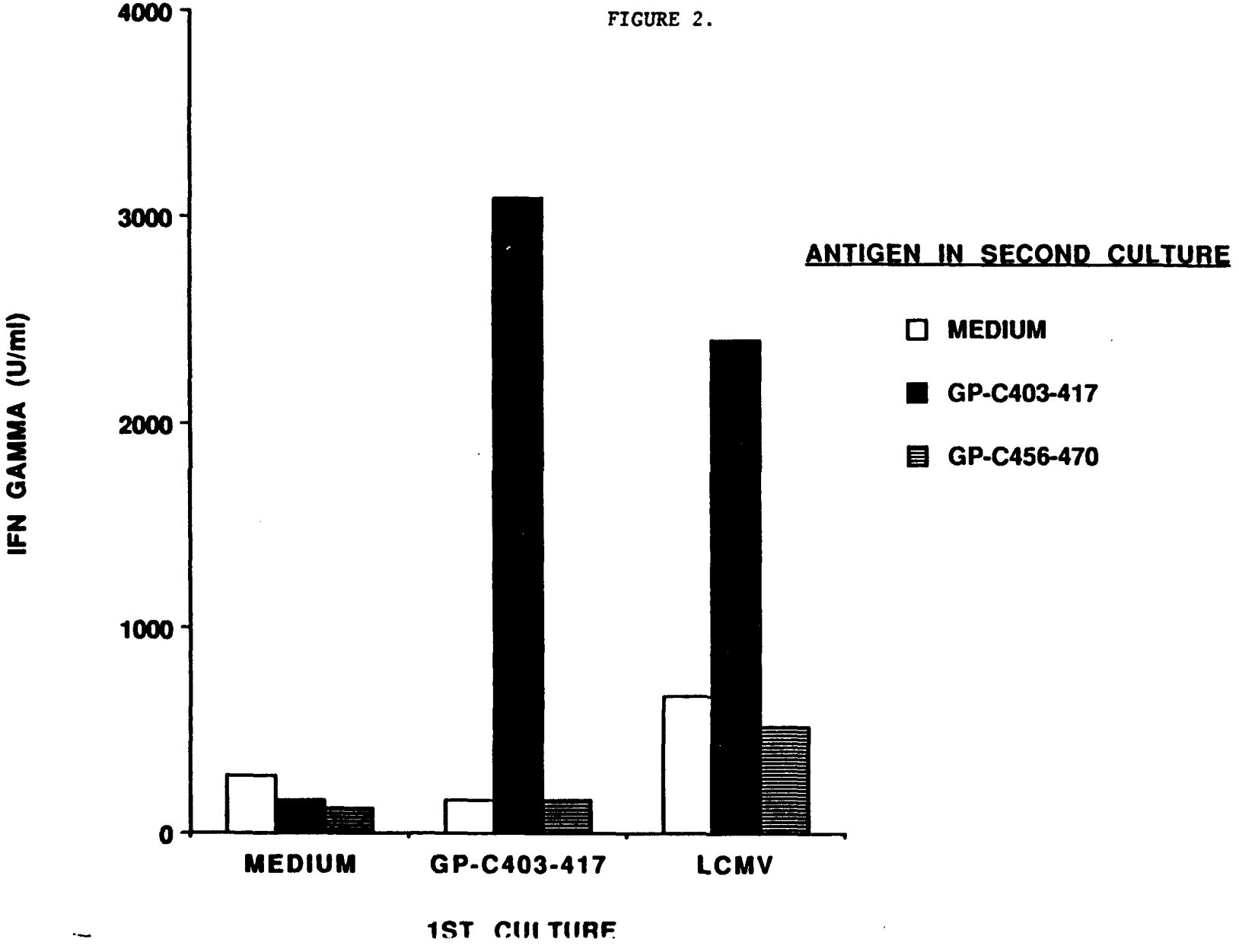

4000
3000
2000
1000
0
IFN GAMMA (U/ml)
MEDIUM
GP-C403-417
LCMV
1ST CULTURE
ANTIGEN IN SECOND CULTURE
MEDIUM
GP-C403-417
GP-C456-470

FIGURE 3

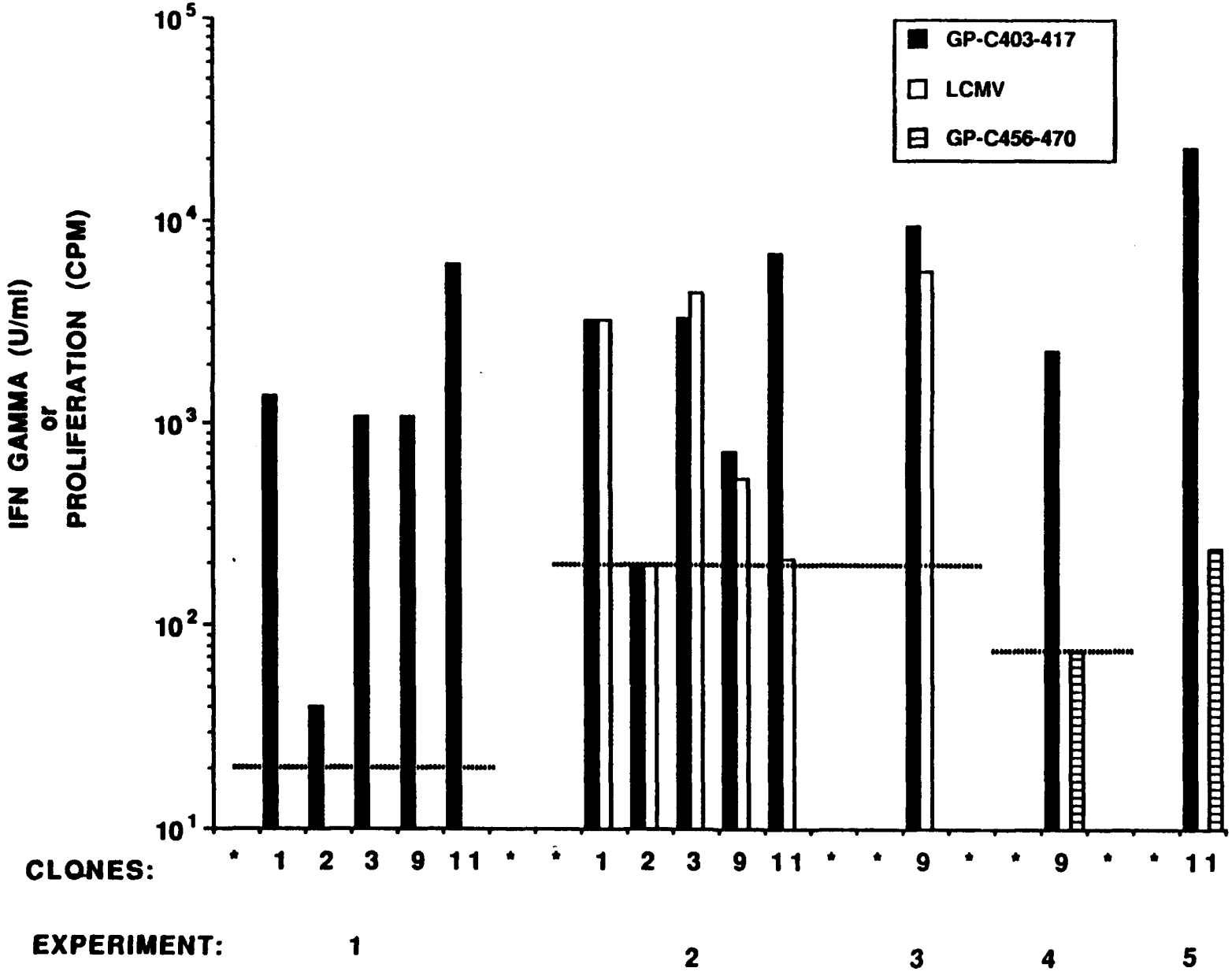

GP-C403-417
LCMV
GP-C456-470
IFN GAMMA (U/ml)
or
PROLIFERATION (CPM)
10^5
10^4
10^3
10^2
10^1
CLONES:
* 1 2 3 9 11 * * 1 2 3 9 11 * * 9 * * 9 * * 11
EXPERIMENT:
1
2
3
4
5

FIGURE 4

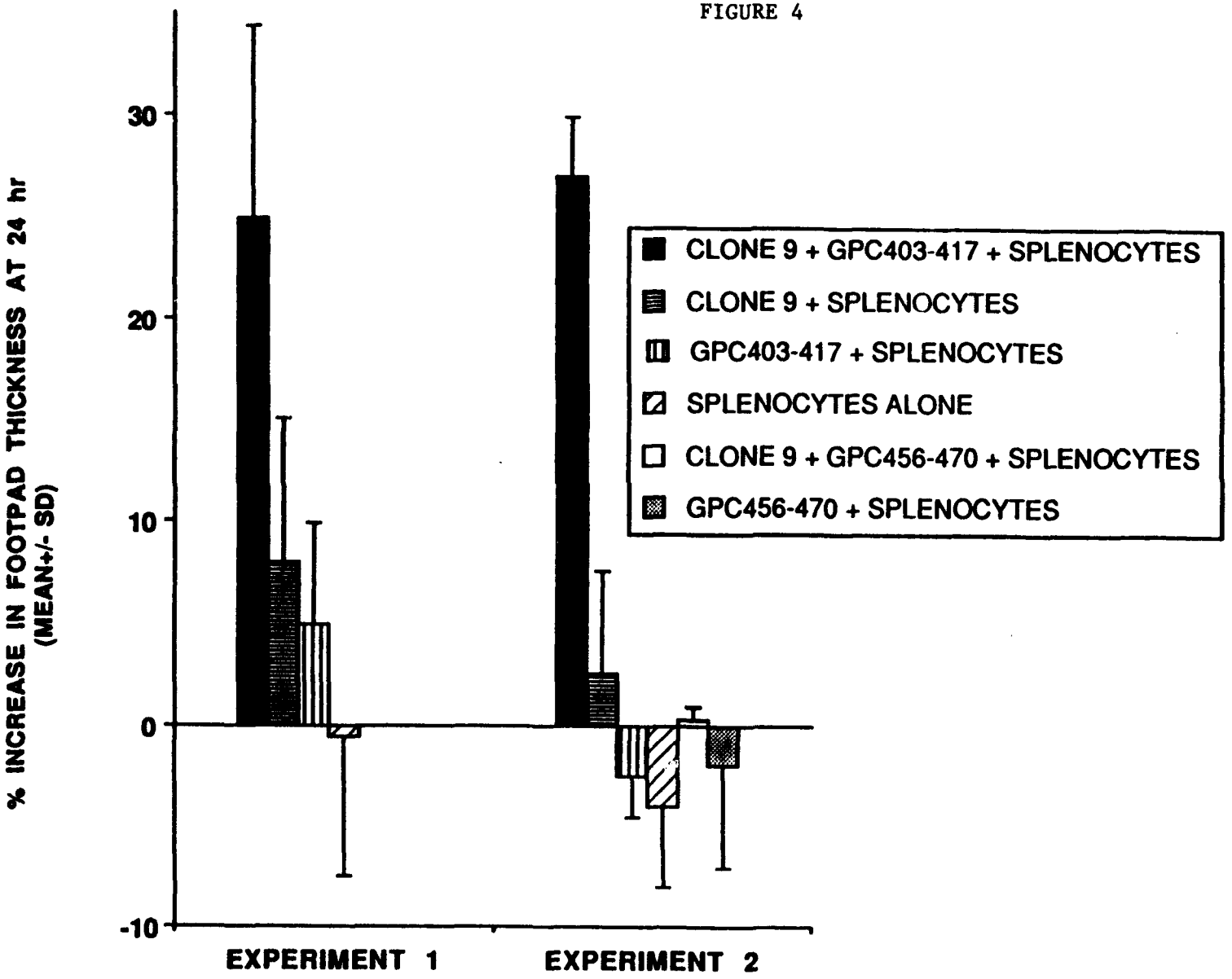

% INCREASE IN FOOTPAD THICKNESS AT 24 hr
(MEAN+/- SD)
30
20
10
0
-10
EXPERIMENT 1
EXPERIMENT 2
CLONE 9 + GPC403-417 + SPLENOCYTES
CLONE 9 + SPLENOCYTES
GPC403-417 + SPLENOCYTES
SPLENOCYTES ALONE
CLONE 9 + GPC456-470 + SPLENOCYTES
GPC456-470 + SPLENOCYTES

Figure 5.

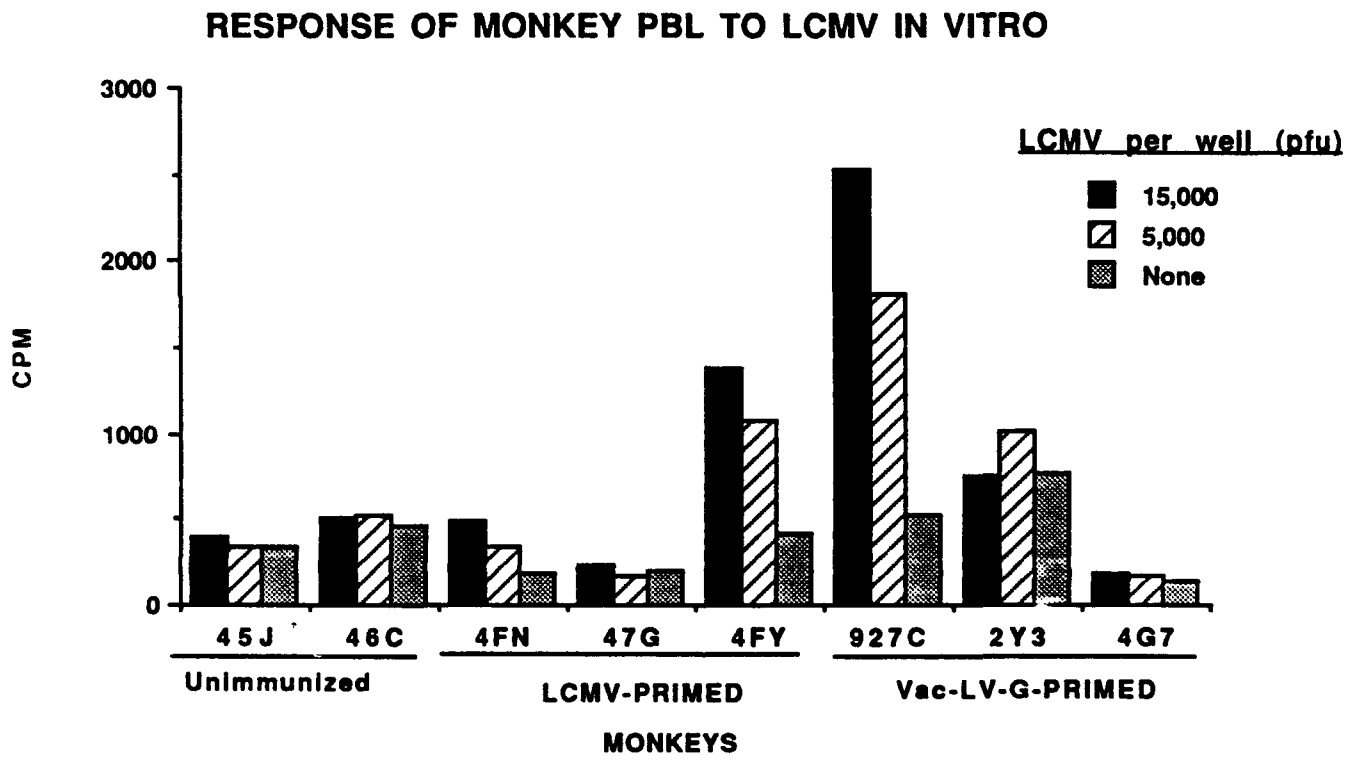
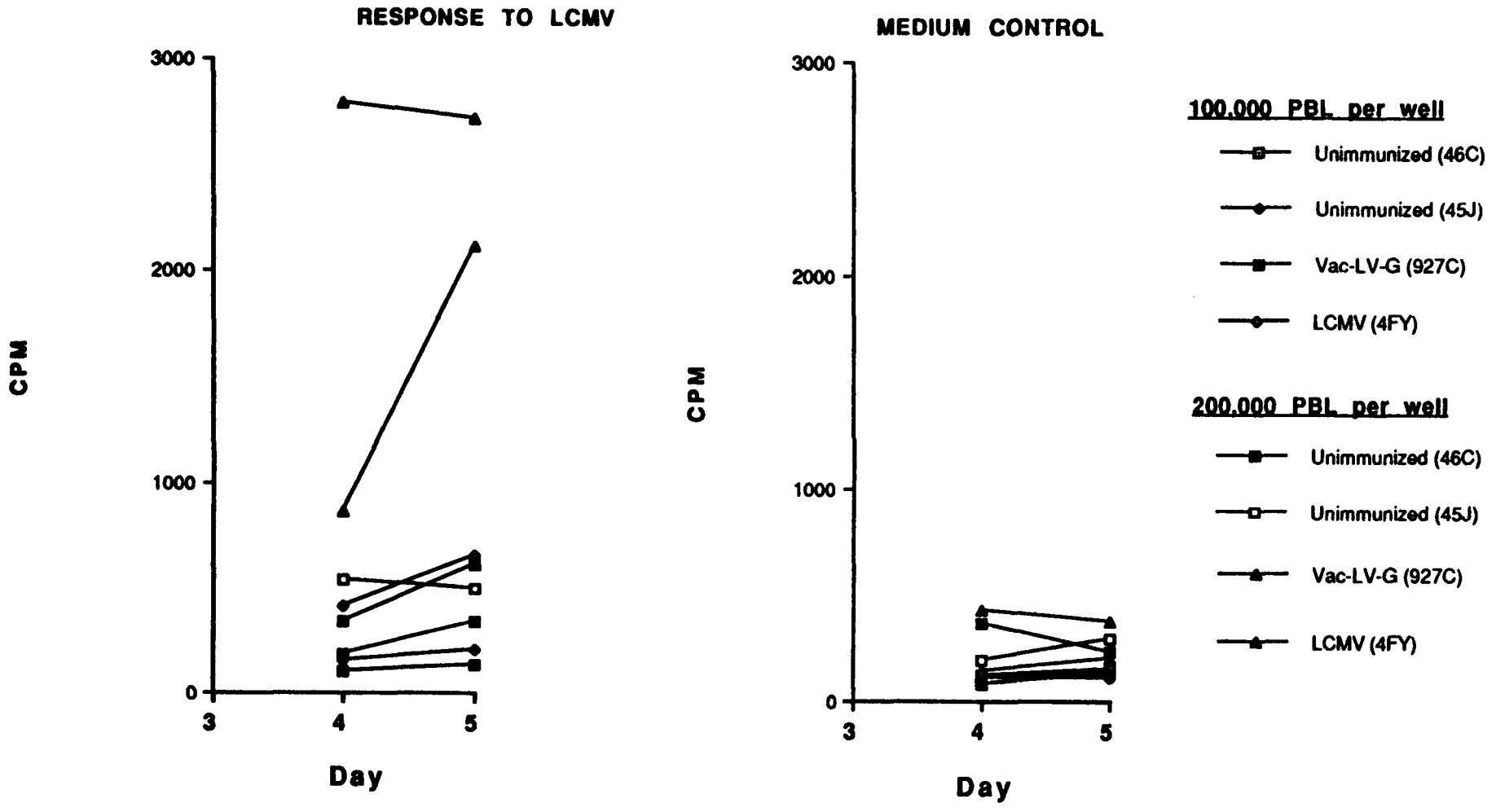

RESPONSE OF MONKEY PBL TO LCMV IN VITRO
CPM
3000
2000
1000
0
LCMV per well (pfu)
15,000
5,000
None
45J
46C
4FN
47G
4FY
927C
2Y3
4G7
Unimmunized
LCMV-PRIMED
Vac-LV-G-PRIMED
MONKEYS

FIGURE 6


RESPONSE TO LCMV
MEDIUM CONTROL
CPM
3000
2000
1000
0
3
4
5
Day
100,000 PBL per well
Unimmunized (46C)
Unimmunized (45J)
Vac-LV-G (927C)
LCMV (4FY)
200,000 PBL per well
Unimmunized (46C)
Unimmunized (45J)
Vac-LV-G (927C)
LCMV (4FY)