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TITLE: IGG SUBCLASS AND ISOTYPE SPECIFIC IMMUNOGLOBULIN RESPONSES TO LASSA FEVER & VENEZUELAN EQUINE ENCEPHALOMYELITIS: NATURAL INFECTION AND IMMUNIZATION

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Venezuelan equine encephalitis (VEE) specific immunoglobulin responses to the two vaccines, TC-83 (a live attenuated vaccine) and C-84 (a formalin inactivated vaccine derived from the TC-83 strain) were evaluated using an antigen and isotype specific enzyme-linked immunoadsorbent assay (ELISA). As previously described, the assays were standardized in methodology and in relation to a uniform reference curve prepared from a pool of subjects with high titers of 80% PR-VNA (80% plaque reduction in the viral neutralization assay). Time-course SERA before and following varying schedules of TC-83 and/or C-84 vaccine were tested suggesting a trend of improved responses to live attenuated vaccine (particularly with IgA and IgM) compared to the killed vaccine. A single dose of TC-83 provided marked antibody responses

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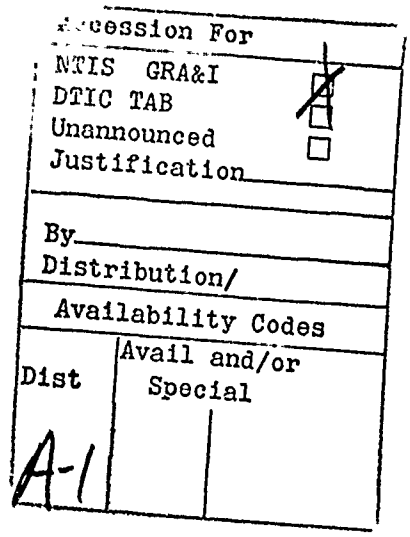
19. Abstract (Continued)

in all isotypes and subclasses except G-2 and G-4. C-84 was an effective booster vaccine except again in terms of IgG4 or G2W with TC-83 plus C84 booster responses combined, VSS specific IgG and IgG_3 correlated best with 80% PR-VNA whereas with TC-83 alone, IgA and IgM correlated best. Lassa-specific ELISA development was complicated by technical difficulties and ongoing.



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RJM Engler 20 aug 90

PI Signature Date

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INTRODUCTION AND BACKGROUND

Immunoglobulin responses to a wide range of infectious agents have been associated with protective immunity, either short or long term. However, in the case of viral infections, virus-specific antibody measurements are not consistently correlated with in vitro neutralization potency or with effective natural immunity. These observed variations may in some cases be due to differences in viral antigens recognized, but they may also reflect differences in isotype or IgG subclass responses that have different efficiency in mediating effective immunity.

Antigen-specific IgG production is generally associated with long-term immunity to a wide range of bacterial and viral infectious agents.(1,2) Human IgG is subdivided into 4 subclasses, each having distinct biologic properties and functions.(1)

VEE, an arthropod-borne RNA virus representative of the alphaviruses in the Togaviridae group, produces epidemic and endemic disease in Central and South America as well as the southern United States. The equine population serves as the principal viremic host but rodents and marsupials can also harbor the virus. Morbidity associated with this disease is considerable but mortality in adults is low (perhaps less than 1%). In children however, the case fatality rate with encephalitis is as high as 35 %. The infection/viremia generates a brisk and promptly effective (neutralizing) antibody response in the host and hyperimmune serum provides effective passive

immunity.(3) The IgG subclass responses to natural infection and vaccination have not been well described for the Venezuelan Equine Encephalomyelitis virus (VEE). The role of IgA and IgM antibody responses to vaccines and in natural infection may explain some of the discrepancies between serologic evidence of viral neutralization but continued risk for infection by the respiratory route.

Two types of vaccines are currently available for the prevention of VEE in man and horses.(4) TC-83, a live attenuated vaccine produced by serial passage of the wild virus utilizing guinea pig fetal heart cell culture, has proved to be efficacious (providing long term immunity) and relatively safe for immunizing horses and man. However, up to 25 percent of individuals vaccinated develop clinical illness with a low grade viremia.(5) In addition, this vaccine may have abortogenic and teratogenic potential and is relatively ineffective in boosting marginal antibody responses.(6)

C-84 is a formalin-inactivated vaccine derived from the TC-83 strain of virus which has been shown to be safe and effective in inducing serum antibody.(4,6) This vaccine produces only mild local and systemic reactions and induces a high titer of neutralizing antibody in both non-immune subjects after 3 immunizations and in sero-positive TC-83 recipients (positive booster effect).(6,7) This vaccine provides effective protection for experimental animals infected by virulent VEE strains by injection but not by aerosol. The mechanism for this is not understood. Vaccinated humans have not been exposed to virulent virus to permit any

conclusions about protective efficacy in man.

Virus specific IgG subclass responses to vaccines may vary with vaccine type and may play a role in the difference in protective potential between the inactivated versus live attenuated vaccines. Selective stimulation of certain subclasses of antibody may be more important in the future when adjuvants and smaller antigens may be used for immunizing agents. In addition, the determination of the IgG subclass predominantly associated with neutralizing antibody could be useful if serum or monoclonal antibodies were to be used for passive immunization against VEE.

The purpose of the study was to develop specific and sensitive assays for the measurement of IgG, IgG subclasses, IgA and IgM directed against VEE antigens. These assays would then be applied to the evaluation of specific antibody responses to the live attenuated (TC-83) and the formalin-inactivated vaccine (C-84). The issue of correlation to the "gold standard", viral neutralization, could then be explored for each of the isotypes and G subclasses. Different groups of subjects who had received different schedules of vaccine would be evaluated utilizing the developed assays in order to determine significant differences.

EXPERIMENTAL METHODS

Human sera positive and negative for VEE specific antibody by viral neutralization assay were obtained through the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), Fort Detrick, Frederick, Maryland. These sera had been previously collected under Human Use Committee approved informed consent including permission for

utilization in other studies as long as the confidentiality of records was maintained.

VEE antigen was also prepared at the USAMRIID facility as follows: TC-83 strain VEE concentrate grown in BHK-21 cell line (Clone 13); gradient purified preparation inactivated by 6 times 10E6 rads of cobalt irradiation. A positive pooled reference serum (with significant high titer 80% plaque reduction on viral neutralization assay) was prepared and utilized for initial testing. Optimum coating concentrations and conditions were tested and subsequently fixed as follows:

1. Dynatech Immulon-2-flat microelisa plates (011-010-3650) were coated with VEE antigen using a carbonate buffer at Ph 9.6 overnight at 4 degrees centigrade in a humidified chamber at 37 degrees centrigade. Plates can be stored coated without loss of of activity for at least a one week period. Each new batch of VEE antigen must be tested with the reference serum in order to decrease inter-assay variability with changes in antigen lots. In addition, storage of aliquoted antigen frozen at minus 70 degrees C. longer than 6 months results in some decay of specific binding at a fixed coating concentration. Coating concentrations varied from a 1:150 dilution to a 1:400 dilution. Assays over time were noted to remain uniform if conditions were always corrected to the same reference serum curve.

   NOTE: Each new batch of VEE antigen must be carefully tested and optimum coating concentrations restandardized for each isotype and

subclass to correct for variations. Our experience found some of the batches to be effective coating antigens at 1:50 dilution only. A pooled reference curve is very useful for this type of restandardization procedure.

2. As described previously, a VEE negative antigen derived from the same culture media as the positive antigen has been utilized to correct for the background binding which was highly variable between individual sera. Alternate columns were subsequently coated with positive and negative antigen, and each serum was run in parallel with at least 4 dilutions on the same plate. Later experiments showed that different batches of VEE negative antigen gave quite similar results in terms of background binding and could therefore be used with any VEE positive antigen batch. We attempted to maintain as much uniformity as possible with batches of negative and positive antigen.

The basic methodology used in the performance of the ELISA's is well described in the Manual of Clinical Laboratory Immunology.(8) Specific reagents tested and utilized during the assay and special procedural considerations are outlined as follows:

1. Sera were initially tested at a dilution of 1:40 with a serial 1:2 dilution on the plate to include at least 4 dilutions. Post vaccine exposure sera were started at 1:160. Later experiments started baseline sera at 1:80 and high titer post vaccine sera at 1:320. Individual subject sera pre and post vaccination were always run in parallel on the same plate in order to decrease intra-assay variability

and optimize titer-fold reproducibility (post divided by pre vaccine titers). Sera were diluted in phophate buffered saline (PBS) with 0.05% Tween-20 and 0.1% bovine serum albumin (0.02% azide as preservative) (PBS-Tween-BSA) and incubated overnight.

2. Detection of specific isotypes and subclasses utilized reagents tested in multiple experiments for specificity (using purified myeloma proteins) and sensitivity. Final selection of the following reagents for the detection antibody included the following: (NOTE: all reagents were diluted in PBS-Tween-BSA and incubated on the plate at least 90 minutes at 37 degrees C.)

   a. Affinity-purified goat anti-human IgG, alkaline phosphatase conjugated (TAGO, Inc., Palo Alto, CA, catalogue no. 4300)

   b. Affinity-purified goat anti-human IgM, alkaline phosphatase (AP) conjugated (TAGO catalogue number 4302)

   c. Affinity-purified goat anti-human IgA, flourescein isothiocyanate (FITC) conjugated (TAGO catalogue number 4201) followed by an affinity-purified goat anti-FITC, AP conjugated (special order RD009 from TAGO, Inc.).

   d. Mouse monoclonal antibodies specific for human IgG subclasses were selected for specificity following testing with a panel of purified G subclass myeloma proteins. The selected antisera are listed below and are currently recognized by the World Health Organization as specific for human IgG subclasses:

      * G-1 (HP 6001); G-2 (HP 6014); G-3 (HP 6050); G-4 (HP 6025).

These reagents were graciously provided by Dr. Reimer of the Center for Disease Control, Atlanta, GA.

3. Subsequent reagents utilized in the G subclass assays included the following:

   a. Affinity-purified goat anti-mouse-FITC conjugated (Coulter catalogue number 6602159) followed by the previously listed anti-FITC.

The intermediate steps between reagents and the final development step with the substrate, p-nitrophenylphosphate (Sigma Chemicals), are well described previously.(8)

Plates were read utilizing an MR-600 Dynatech Microflour reader utilized in conjunction with an Apple IIe computer and the software Immunosoft version 2.4. VEE antigen negative (VEE -) optical densities were subtracted from VEE positive (VEE +) antigen binding in parallel dilutions. The negative binding was significantly above plate/reagent background for the IgG, IgA, IgM, and IgG-2 assays but not for G-1 or G-3 (or G-4). Each assay was standardized to the uniform reference curve and both end-point titers and units/ml (based on assigned units to the reference curve) were calculated using log-logit transformations and curve fitting.(9)

Viral neutralization assays specific for VEE are performed routinely at the USAMRIID laboratories and 80% plaque reduction/conversion titers for each of the sera studied were provided courtesy of J. Mangiofico.

RESULTS

Appendix 1 through 6 (A1-A6) summarizes the geometric mean data for VEE

specific IgG, IgA, IgM and the IgG subclasses in two groups of laboratory workers: Group A (subjects who recieved a single dose of TC-83 vaccine, N=20) and Group B (subjects with a history of exposure to a TC-83 vaccine dose, loss of protective immunity by viral neutralization assay, who received a single booster dose of C-84 vaccine, N=19). Appendix 1 and 3 tabulate the geometric mean titers (with standard errors of the mean) for VEE specific IgG, IgG subclasses 1 through 4, IgA, and IgM along with the 80% PR-VNA mean titer. There is no significant difference between the pre mean titers for each of the isotypes, subclasses or PR-VNA except for VEE specific IgG3. Appendix 2 is a graph of the mean titers tabulated in A1. Appendix 4 is a bar graph of the mean post immunization titers with a significant difference demonstrated for the IgM isotype and marginally ($P = 0.49$) for IgA. Appendices 5 and 6 summarize in table and bar graph form, the geometric mean titer-fold increases (3 week post divided by pre vaccination titers) with the most significant difference for the IgM isotype and only a marginal difference for IgG.

When the 80% plaque reduction viral neutralization assay titer increases were correlated to the different isotypes and subclasses within each of the two groups, it was noteworthy that IgA and IgM correlated best for the TC-83 group (R values of 0.46 and 0.57 respectively with P values less than 0.04 and 0.008). IgG and IgG3 correlated best for the C-84 booster group (R values of 0.63 and 0.46 respectively with P values less than 0.03 and 0.05).

Additional testing of time sequenced sera in 6 subjects who received 2 doses of C-84 vaccine demonstrated markedly decreased IgA responses with

4 of the six subjects demonstrating a less than 4 fold titer increase after even the second booster of vaccine.

Earlier development studies had detected one subject of Asian background who had a very high VEE specific IgG2 titer in contrast to a majority of other subjects tested. Eight additional sera from subjects of Asian heritage were tested but not found to have a consistent response in the IgG2 subclass.

CONCLUSIONS

A single exposure to the live-attenuated Venezuelan equine encephalitis vaccine TC-83 generates a brisk antibody response with protective titers as measured by the plaque reduction viral neutralization assay. This response is paralleled in the IgG, IgA and IgM response with the IgG represented by IgG1 and IgG3 subclasses. No IgG4 was observed and IgG2 booster responses are generally low with less than a four fold increase in a majority of patients. In subjects with a prior exposure to TC-83 but loss of protective titers by 80% PR-VNA, the killed vaccine C-84 provides an effective booster response in essentially the same antibody distribution. The IgG3 booster response was most prominent with the C84 booster and paralleled the 80% PR-VNA.

The observation that the 6 subjects receiving C-84 vaccine only (1 or 2 boosters) did not mount a significant IgA response is of interest because of earlier observations that protection against aerosal infection with VEE was not consistent in hamsters that had only received C-84 inactivated vaccine.(9) IgA is the dominant secretory antibody providing protection

at mucosal surfaces. Virulent VEE is considered a neurotropic virus, and it exhibits significant infectivity via the respiartory tract. The potential of VEE virus to invade the central nervous system via the cribriform plate has been documented for nonhuman primates (10,11), and in hamsters VEE virus has been shown to invade the olfactory bulb.(12,13) Mucosal immunity may prevent invasion of the cribriform plate. IgA may be a critical isotype for this type of protection. Live replicating antigen is superior to inactived antigen in local immunity in other systems such as measles (14), and this may be associated with the differences in stimulation of IgA secretion.

Subsequent work will focus on additional testing of sera from subjects having received the inactivated C-84 vaccine with comparison to earlier parts of the study. In addition, attempts to obtain sera from subjects who developed laboratory infection despite an antecedent exposure to vaccine may be of interest to compare with the different vaccine groups.

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TABLE OF GEOM. MEANS (OF TITERS)

PRE VACCINES WITH STANDARD ERROR OF MEAN ()

VEE SPECIFIC AB	GRP-A n=20	GRP-B n=19
80% PR-VNA	0	0
G-TOTAL	110(1.70)	116(2.50)
G-1	43(1.91)	82(3.20)
G-3	52(2.29)	39(2.00)
G-2	102(2.31)	90(2.17)
A	80(2.04)	69(2.85)
M	119(2.85)	155(2.17)
G-4	NONE	NONE

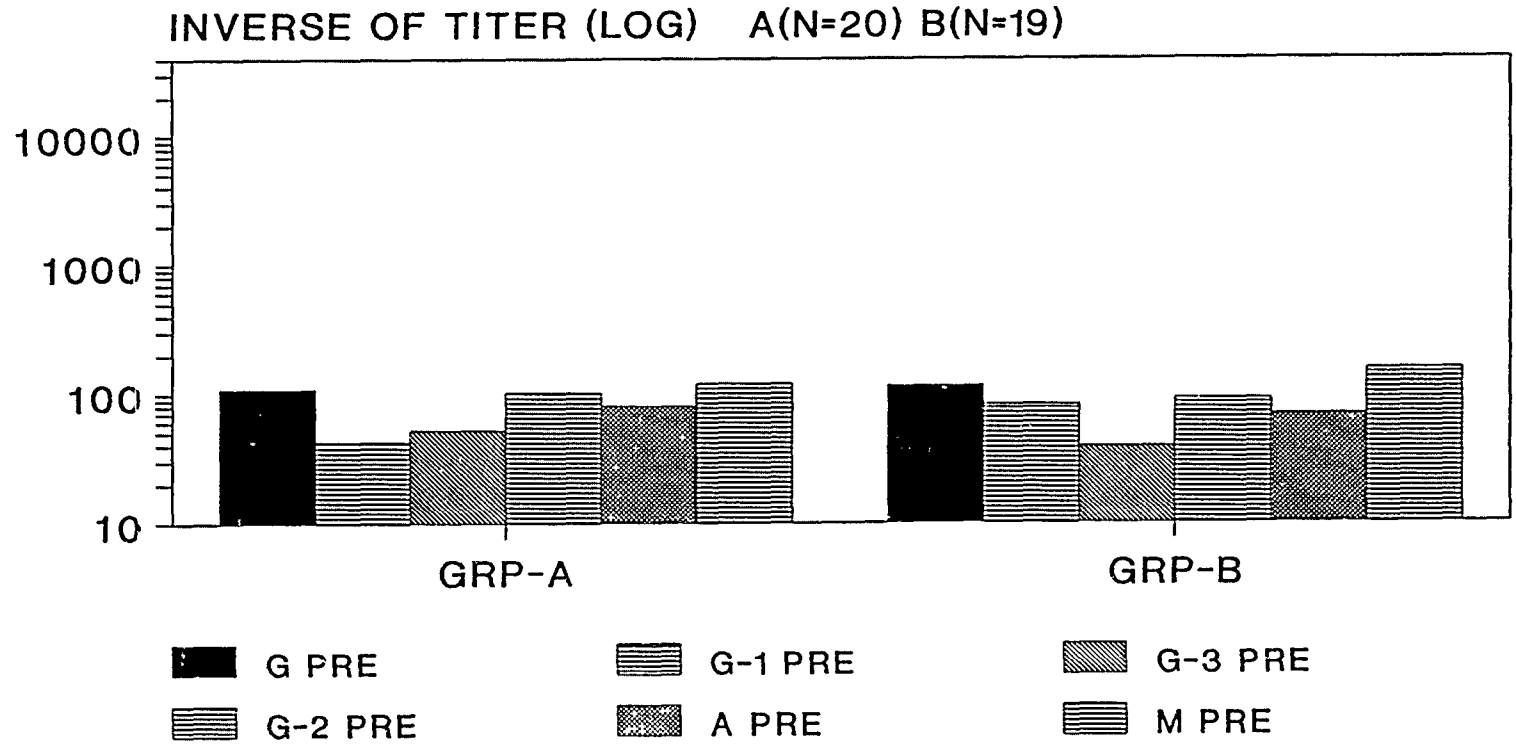
80% PR-VNA: 80% PLAQUE REDUCTION IN VEE SPECIFIC VIRAL NEUTRALIZATION ASSAY

TC83 ONLY (GRP-A); TC83/C84 BOOSTER (GRP-B)

PRE VACCINATION VEE-SPECIFIC AB:

GEOMETRIC MEANS

TC83 ONLY (GRP-A) VS TC83/C84 (GRP-B)



80% PLAQUE REDUCTION TITERS (PR-VNA) = 0
ELISA TITERS BASED ON O.D. (2 X BKGD)
AFTER SUBTRACTION OF NEGATIVE AG BINDING

Appendix II

TABLE OF GEOM. MEANS (OF TITERS)

POST VACCINES WITH STANDARD ERROR OF MEAN ()

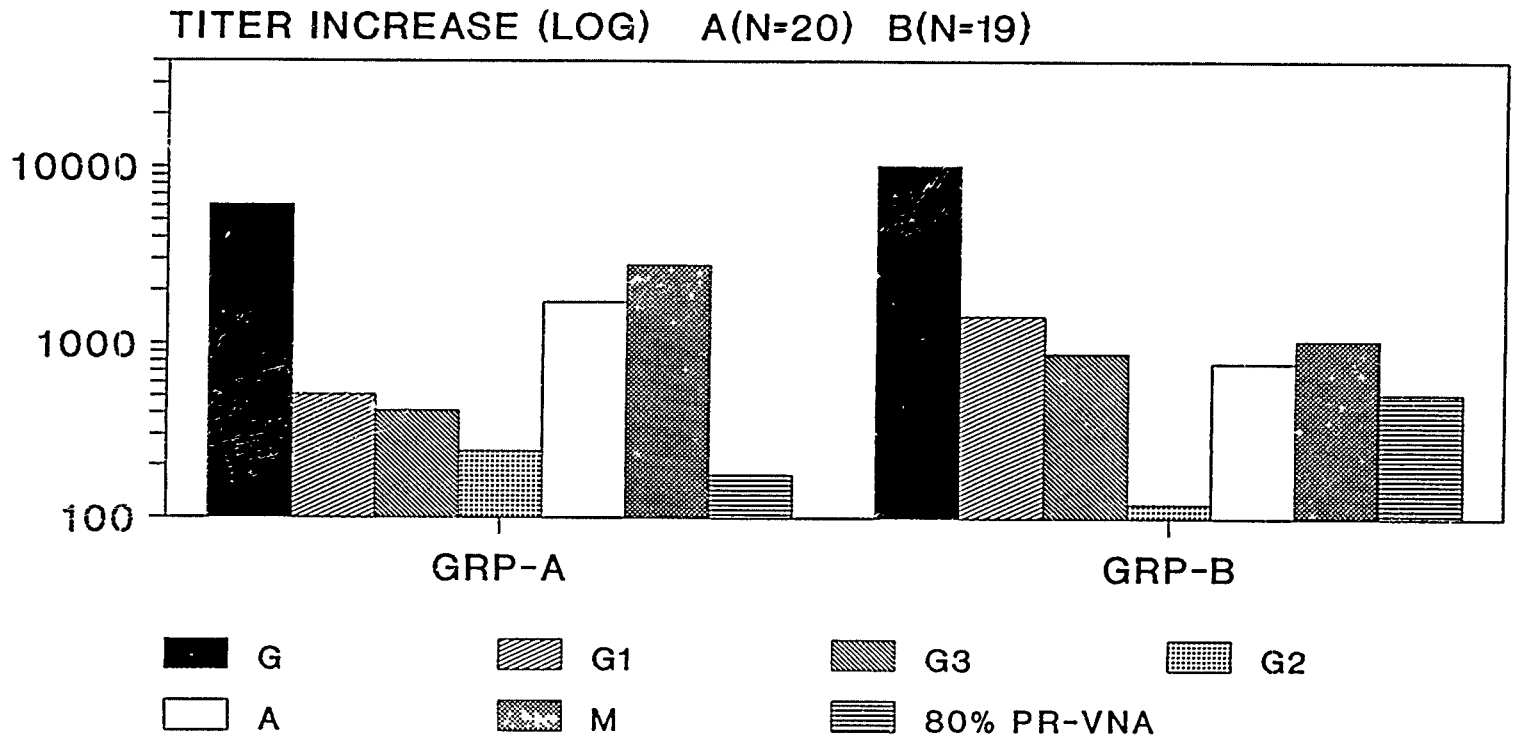
VEE SPECIFIC AB	GRP-A n=20	GRP-B n=19
80% PR-VNA	175(3.84)	517(6.48)
G-TOTAL	6073(2.77)	10205(4.57)
G-1	509(2.60)	1442(4.55)
G-3	414(3.33)	886(7.15)
G-2	240(3.80)	121(3.09)
A	1709(2.09)	779(3.01)
M	2777(2.68)	1052(4.74)
G-4	NONE	NONE

80% PR-VNA: 80% PLAQUE REDUCTION IN VEE SPECIFIC VIRAL NEUTRALIZATION ASSAY

TC83 ONLY (GRP-A); TC83/C84 BOOSTER (GRP-B)

Appendix III

GEOMETRIC MEANS (GM) COMPARED:
VEE SPECIFIC TITERS POST VACCINES
TC83 ONLY (GRP-A) VS TC83/C84 (GRP-B)



P < 0.05 FOR A VS B (A & M)
POST-VACCINE TITER/PRE-VACCINE TITER
NOTE: G1 P=0.03 BUT PRE'S DIFF P=0.03

Appendix IV

TABLE OF GEOM. MEANS (WITH SEM)
OF TITER-FOLD INCREASES (POST/PRE TITER RATIO)

VEE SPECIFIC AB	GRP-A n=20	GRP-B n=19
80% PR-VNA	175(3.84)	517(6.48)
G-TOTAL	55(3.20)	87(5.43)
G-1	12(3.42)	18(5.21)
G-3	8(3.45)	23(9.78)
G-2	2(2.99)	1.3(2.35)
A	21(2.78)	11(4.33)
M	23(2.95)	7(5.35)
G-4	NONE	NONE

80% PR-VNA: 80% PLAQUE REDUCTION IN VEE SPECIFIC VIRAL NEUTRALIZATION ASSAY

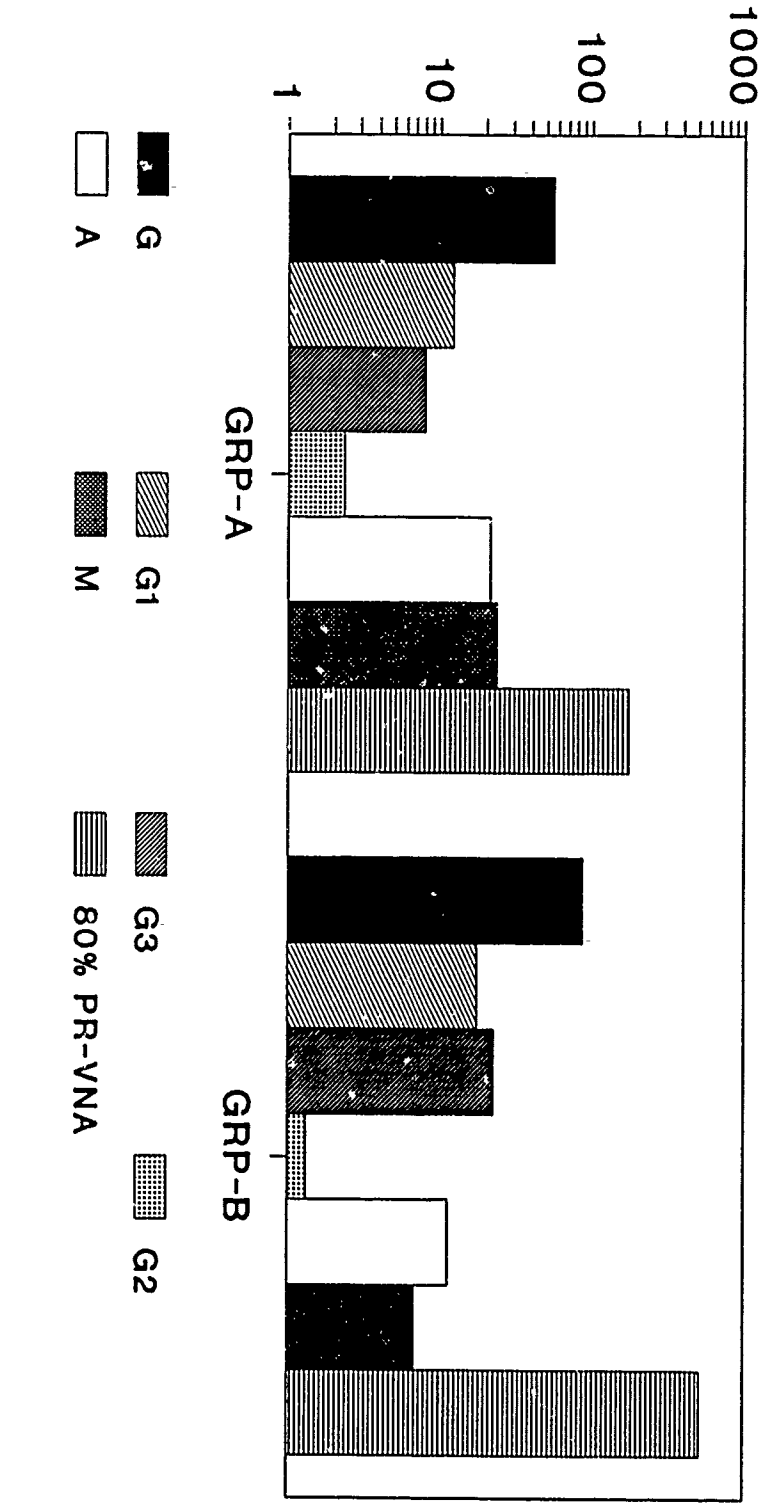
TC83 ONLY (GRP-A); TC83/C84 BOOSTER (GRP-B)

Appendix VI

GEOMETRIC MEANS (GM) COMPARED:

VEE SPECIFIC TITER-FOLD INCREASES

TC83 ONLY (GRP-A) VS TC83/C84 (GRP-B)



P < 0.05 FOR A VS B (A & M)
POST-VACCINE TITER/PRE-VACCINE TITER
NOTE: G3 P=0.06(LOG); P=0.04 (NON-LOG)

LASSA SPECIFIC ANTIBODY ASSAY

Lassa Fever (LF), one of the rodent borne Arenaviruses producing acute hemorrhagic fever in man, can cause a mild to severe and fatal (in up to 20% of cases) systemic disease as a result of microvascular damage and changes in vascular permeability.(1,2) The virus is found predominantly in West Africa but is related to the Argentine and Bolivian hemorrhagic fevers (Junin and Machupo) found in South America.

Immunologically, this disease is associated with late appearance of neutralizing antibody, and serum from the early convalescent phase of the disease does not provide passive immunity.(3) In contrast, sensitized spleen cells have been shown to provide passive protection. No effective vaccine is currently available and this virus is representative of a group of RNA viruses that generate an initial antibody response with natural infection that does not provide effective immunity. At the same time, in the primate model, passive administration of neutralizing antibody can significantly decrease mortality particularly when administered early in the disease course or in combination with antiviral agents such as ribavirin.(4,5) Also of interest with this disease, the quantitative levels of viral specific antibody are not significantly different between the early and late convalescent phase of the disease, yet neutralizing capability of this antibody is significantly different.(5) It is possible that this difference is due to the virus peptide specificity of the antibody or to its avidity. However, Western blot studies have shown that non-protective early

convalescent antibody does react with all three virion peptides. (Jahrling, unpublished observations) Thus, this difference may be related to IgG subclass differences; if so, plasma screening of G-subclass specific antiviral antibody may be useful in the selection of optimum donors for the preparation of hyperimmune globulin. In addition, the efficacy of an antiviral monoclonal antibody may be dependent on the subclass created.

A panel of sera from Sierra Leon have been collected from the serum bank of USAMRIID; these sera are representative of subjects who had been infected with the Lassa virus or had no history of exposure and no neutralizing antibody. These sera were used to prepare a positive pooled sera as a reference and provided a panel of negative control sera. Multiple batches of inactivated Lassa antigen were tested using passive coating techniques, special blocking techniques and inhibition strategies in order to determine isotype and IgG subclass responses. Unfortunately after a series of experiments, it has become clear that as described in reference 6, this may not be a feasable approach and a supply of antigen capture antibody will be required in order to proceed with this phase of the study in the future.

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